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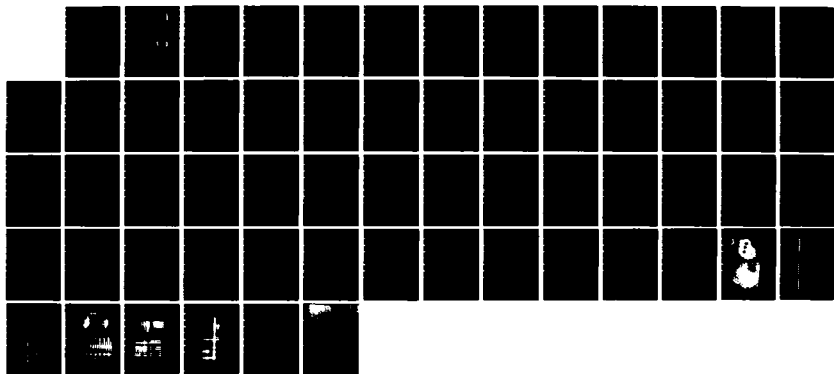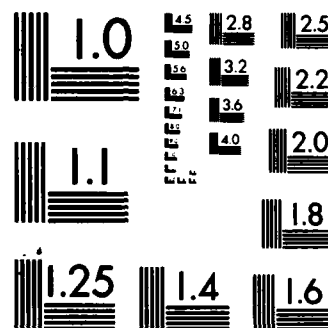UNCLASSIFIED   F/G 6/5   NL

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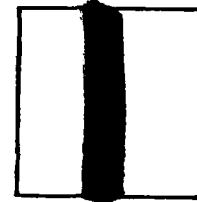
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In vitro Studies of Sandfly Fever Viruses and Their Potential
Significance for Vaccine Development

Annual Progress Report

by

Jonathan F. Smith, Ph.D.

February, 1981

(for the period 1 January, 1980 - 1 January, 1981)

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The structural and nonstructural polypeptides synthesized by several sero-logically distinct strains of sandfly fever viruses have been studied by immuno-precipitation and SDS-polyacrylamide gel electrophoresis. The resulting profiles show that a similarity exists in the number and molecular weights of the major structural and nonstructural proteins, but that most strains are distinguishable by this procedure. A similar analysis of six strains of Punta Toro virus which differ markedly in their virulence for laboratory animals has demonstrated that differences are not detectable in internal or nonstructural polypeptides, but

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that minor differences are seen in the surface glycoproteins. Analysis of the envelope proteins of Punta Toro and Karimabad viruses synthesized in the presence of tunicamycin has indicated that this technique allows the resolution of polypeptides which are not resolved in their glycosylated state, that the 2 envelope proteins are glycosylated to different extents, and that the sum of the molecular weights of the nascent polypeptides is equivalent to that of a high molecular weight protein which is immunoprecipitated from infected cells treated with zinc ions.

The antigen specificities of a panel of monoclonal antibodies produced against Punta Toro virus have been determined by immunoprecipitation and acrylamide gel analysis. Most of the monoclonal antibodies recognize determinants present on the nucleocapsid protein, however, four react against determinants on GP-2, and one against determinants on GP-1. Evidence is presented which suggests that each of the GP-2-specific hybridomas bind to different antigenic determinants.

RNA extracted from Karimabad virus-infected cells and added to rabbit reticulocyte lysates directs the synthesis of the major nonstructural protein (31K) and the nucleocapsid protein (24K), as well as several other proteins which have not been detected in infected cells. One of these proteins (48K), however, is also detected in infected cells inhibited with zinc. High molecular weight proteins (greater than 100K) are also detected in in vitro translation experiments and zinc-inhibited cells. The data from these two experiments have been used to construct a tentative scheme for the synthesis of virus-specific polypeptides which is based, in part, on the cleavage of precursor proteins.

Data are also presented which indicate that adult mice are susceptible to the prototype strain of Punta Toro virus if inoculated intracerebrally, and that this system can be used to evaluate the relative biological roles of individual viral proteins. Using this model, we have shown that a monoclonal antibody to a (single) antigenic determinant on GP-1 is sufficient to provide protection if passively transferred before or after virus challenge, whereas the existing monoclonal antibodies directed against GP-2 provided no protection. Recent data has indicated that an alternate strain of Punta Toro virus is capable of inducing lethal infections in C57BL/6J mice following peripheral inoculations (intraperitoneally or subcutaneously), and that this may be a more advantageous model in which to pursue additional studies.

CONTENTS

I. Abstract

The structural and nonstructural polypeptides synthesized by several serologically distinct strains of sandfly fever viruses have been studied by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The resulting profiles show that a similarity exists in the number and molecular weights of the major structural and nonstructural proteins, but that most strains are distinguishable by this procedure. A similar analysis of six strains of Punta Toro virus which differ markedly in their virulence for laboratory animals has demonstrated that differences are not detectable in internal or nonstructural polypeptides, but that minor differences are seen in the surface gly- coproteins. Analysis of the envelope proteins of Punta Toro and Karimabad viruses synthesized in the presence of tunicamycin has indicated that this technique allows the resolution of polypeptides which are not resolved in their glycosylated state, that the two envelope proteins are glycosylated to different extents, and that the sum of the molecular weights of the nascent polypeptides is equivalent to that of a high molecular weight protein which is immunoprecipitated from infected cells treated wiht zinc ions.

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II. Underline: General Introduction

Among the over 400 viruses which are presently classified as arboviruses, approximately 180 belong to the bunyavirus genus or are considered bunyavirus-like, and at least one-fourth of these are known to cause significant human disease. Thus, collectively, bunyaviruses constitute the largest family of arthropod-borne viruses, and the largest grouping among the Bunyaviridae, the phlebotomus or sandfly fever viruses, will be the subject of this report. Despite their importance as human or animal pathogens, the Bunyaviridae remain the least well understood of the major arbovirus groups in terms of their basic virology and biology, the replication patterns which they establish in infected cells, and with respect to the approaches which should be considered in the design of experimental vaccines.

There are currently 31 serologically distinct viruses which have been placed in the sandfly fever virus group on the basis of reciprocal HI reactions, and an additional three recent isolates are also being considered for inclusion within this group. In addition to two of the three new isolates, there are six strains which are known to be pathogenic for man. Three new-world viruses, Punta Toro, Chagres, and Candiru viruses produce symptoms similar to the old-world strains of Naples and Sicilian viruses. These diseases are incapacitating, similar in many respects to dengue fever, but are self-limiting and nonlethal. The sixth virus, Rift Valley Fever virus, which has only recently shown to be a member of the phlebotomus group, induces a more serious disease with complications of encephalitis, hemorrhagic fever and retinal lesions, with approximately 5% mortality. Our studies have included all of the phlebotomus viruses known to be human pathogens (as well as other members of this group), with the exception of Rift Valley Fever virus which requires high level containment. However, for reasons which are described within the body of this report, our experiments have focused on Punta Toro and Karimabad viruses which possess molecular or virulence characteristics which make these strains particularly desirable in the study of specific aspects of sandfly fever replication in vitro or in vivo.

As has been defined in previous proposals, the objectives of our studies are 1) to describe for this group of viruses the virus-coded structural and nonstructural proteins, their interrelationships, and post-translational processing, 2) to describe the mechanisms by which these viruses control the expression of their genomes, i.e., whether these viral proteins are translated from independent or polycistronic messengers, 3) to correlate these biochemical data to those obtained from morphology and morphogenesis studies based on electron microscopy studies which have been completed, and 4) to initiate animal studies in which the biological roles of these viral proteins, which have been identified and characterized in in vitro experiments, could be evaluated in vivo (specifically, to determine which viral antigens and antigenic determinants are capable of providing immunologic protection), and 5) to collaborate in the production and characterization of monoclonal antibodies to Punta Toro virus proteins which are of use in both in vivo and in vitro experiments.

The data which are presented in the body of this report represent the progress which has been made in the current year with respect to these objectives, and are discussed at length within. The major findings may be summarized as follows:

1) The analysis by gel electrophoresis of the structural and nonstructural polypeptides of 12 strains of sandfly fever viruses has shown that, although striking similarities exist in these profiles, most strains are distinguishable by this technique.

2) In a similar analysis of six different strains of Punta Toro virus which differ widely in their relative virulence for laboratory animals, minor, but reproducible, differences are seen in the migration of viral glycoproteins.

3) Tunicamycin has been used to further study the glycosylation patterns of the envelope proteins of Punta Toro and Karimabad viruses and to determine the size of the nascent, unglycosylated polypeptides. These data were required to interpret the nature of high molecular weight polypeptides detected in experiments summarized below.

4) Immunoprecipitation analysis of monoclonal antibodies produced by 99 clones of hybridomas secreting antibody to Punta Toro virus antigens has shown that most are reactive to the nucleocapsid protein, but that four recognize determinants on GP-2 and one reacts with GP-1.

5) Preliminary tryptic peptide analysis of the structural and nonstructural polypeptides of Karimabad virus has suggested that the nucleocapsid protein may share limited amino acid sequence homology with the major nonstructural protein.

6) Using zinc ions as a protease inhibitor, polypeptides have been identified in both Karimabad and Punta Toro virus-infected cells which may be precursors to the mature envelope proteins. A lower molecular weight polypeptide has also been detected in zinc-inhibited cells infected with Karimabad virus which may be a precursor to the nonstructural polypeptide.

7) In in vitro translation of RNA from Karimabad virus infected cells, several apparently virus-specific proteins have been observed, some of which have not previously been detected in immunoprecipitates from intact cells. Based on these results and those from zinc-inhibition studies, a tentative model for the synthesis of sandfly virus protein synthesis and processing has been proposed, which is subject to experimental confirmation, and encompasses a significant proportion of the renewal application.

8) Infection experiments have been initiated with Punta Toro virus in mice to evaluate the role of Punta Toro virus antigens and antigenic determinants in providing protection. Data will be presented to describe two possible models which will be useful to pursue these studies. Preliminary data are also shown which indicate that antibody to a single determinant on GP-1, passively transferred, provides absolute protection from an otherwise lethal challenge, whereas other antibody preparations are ineffective. This animal model is also proposed in the accompanying application to further evaluate, by passive and active immunization, the relevant antigens desired in, and the relevant immunoresponse desired from, experimental immunogens.

III. General Methods

Specific procedures for RNA extraction, in vitro translation, characterization of monoclonal antibodies, tryptic peptide mapping, and treatment of cells with zinc or tunicamycin are included in the relevant sections below.

A. Immunoprecipitation

Subconfluent monolayers of either BHK or vero cells were infected with sandfly fever viruses at a multiplicity of infection of 0.1 to 10 (as indicated). At appropriate times (usually 14-18 hrs) after infection the cells are labeled with 50 microcuries of tritiated leucine per ml in leucine-free media or 200 microcuries per ml of 2-^{3}H-mannose. At the end of the labeling period the cells are removed from the surface and lysed in buffer containing 1% triton X-100, 0.02M tris-HCl buffer (pH 7.5), 0.05 m NaCl and 5 micromolar PMSF (phenylmethane sulphonyl fluoride, protease inhibitor). The cells are incubated in this buffer for 15 minutes at 4°C, gently homogenized, and the nuclei (except where noted) are removed by centrifugation, DOC is then added to a final concentration of 0.5% and the lysate is centrifuged for 2 minutes at 15,000 RPM in a Brinkman microfuge. Viral proteins can then be selected from this lysate by either 1) direct precipitation, 2) indirect precipitation using staphylococcal protein A, or 3) immunoaffinity chromatography. Immunoaffinity chromatography, in which antibody is covalently linked to agarose beads (see 1980 annual report) has been used for preparative procedures, but is not practical for the analysis of large numbers of samples.

Direct precipitation has been found to give the lowest background of host cell polypeptides, but various dilutions of HMAF must be added to each lysate sample to assure equivalence point precipitation, thus limiting the number of samples which can be analyzed and increasing greatly the amount of immunoglobulin required. We have found that indirect precipitation using protein A (protein A-sepharose, Pharmacia; or Pansorbin, Calbiochem) is the most efficient of the three procedures in precipitating viral proteins and is now used routinely. For analytical purposes, cells are grown in 24 well plates (Falcon), each well having a 2 cm^2 surface area containing 1-2x10^5 cells. Following infection and labeling as described above, 100 microliters of lysis buffer is added to each well and the clarified lysate prepared as described above. Fifteen microliters of specific HMAF is then added to the lysate and incubated from 4 hours to overnight at 4°C. Protein A-sepharose is then added, the lysate is incubated for 10 minutes at 4°C, and the agarose beads with the adsorbed immune complexes are pelleted. Following 3 serial washings of this complex in lysis buffer, the final pellet is solubilized and prepared for SDS-gel electrophoresis.

Alternatively, these immune complexes can be prepared for isoelectric focusing and 2-dimensional gel analysis by dissolving the precipitate in 2% triton X-100, 8 M urea, and 5% mercaptoethanol. These compounds free the precipitated antigens, are compatible with isoelectric focusing procedures, and must be included in the focusing gels, with the exception of mercaptoethanol which inhibits polymerization.

B. Polyacrylamide gel electrophoresis

Discontinuous gel electrophoresis was carried out in a 28 cm slab gel apparatus (Bio-Rad L boratories, Richmond, Cal.) using a discontinuous system modified from L emmli   ). DATD (N, N'dialytartardiamide) was used as a cross-linker

rather than Bis-acrylamide due to its better resolution of glycoproteins. The ratio of acrylamide to DATD was 30:1.6, and the stacking gel and the resolving gel were 5% and 13%, respectively. The sample buffer contained 2% sodium dodecyl sulfate (SDS), 5% mercaptoethanol, 12% glycerol, 0.005 M phosphate buffer (pH 7.0), and 5-10 μM phenylmethylsulphonyl fluoride (PMSF) as a protease inhibitor. All reagents were purchased from Bio-Rad Laboratories. Samples were dissolved directly in sample buffer (preheated to 100°C) and boiled for 2 minutes. Electrophoresis was carried out using constant voltage (60-90v) for 14-18 hours. The gels were then fixed and stained with coomassie brilliant blue or prepared directly for fluorography.

C. Fluorography

To allow autoradiographic presentation of tritium-labeled gel profiles, gels were impregnated with PPO, according to the procedure of Lasky and Mills (2). Gels were then dried onto filter paper or cellophane and exposed to Kodak BB-5 x-ray film at -70°C. Fluorographic enhancement was also used for ^{35}S-methionine-labeled polypeptides synthesized in in vitro translation reactions.

IV. RESULTS AND DISCUSSION

1) Fine Structure and Protein Topology of Karimabad Virus

As indicated in the previous proposal, one of the objectives of our studies with sandfly fever viruses has been to define the role of various viral proteins in the morphogenesis of these viruses, the sequence of protein interactions which culminate in budding and assembly, and the organization of proteins in the mature virion. Several observations have suggested that these processes may differ significantly when compared to other enveloped, negative-stranded (NS) viruses. First, we have shown by thin-section analysis of cells infected with several sandfly viruses, that the interaction of viral RNP with viral glycoproteins takes place initially at membranes of the Golgi complex, where envelope acquisition occurs, rather than at the plasma membrane which is usual for NS-RNA viruses. This seems to be a property shared by most, if not all, bunyaviruses (3). Second, these viruses lack an M-type protein which is critical in the morphogenesis of all other NS-RNA viruses (with the exception of Arenaviruses), being essentially a connecting protein between viral RNP and envelope proteins. Thus, it was originally suggested that at least one of the sandfly virus glycoproteins must be transmembranal, at least during budding. Data suggesting that this is indeed the case has been presented in the previous report in which chymotrypsin treatment of isolated cytoplasmic vesicles resulted in the removal of a specific 8,000 dalton fragment (presumably the carboxyl terminus) from Karimabad glycoprotein(s). Third, the glycosylation of Punta Toro virus and Karimabad virus glycoproteins is unique among enveloped viruses insofar as only simple (high mannose) oligosaccharides are present - at least on intracellular glycoproteins immunoprecipitated from infected cell lysates.

It was also considered possible that studies in which the roles of various viral proteins in virus maturation were evaluated may indicate a function for the 31,000 molecular weight nonstructrual protein which has now been detected in cells infected with several sandfly viruses (in addition to Karimabad) whose function is presently unknown. Experiments were also suggested in which the orientation of viral proteins in mature virions and in maturation complexes extracted from infected cells would be determined relative to each other and relative to the Golgi/virion membrane. These studies are currently underway and are using surface-specific (iodination) labeling, bifunctional (and cleavable) cross-linkers of known reaction radii, and proteases of high specific activity (carboxypeptidase and aminopeptidase). However, a necessary baseline for all of these studies is that the morphology and protein orientation/interaction in mature virions be firmly established. Although seemingly straightforward, these studies have been surprisingly difficult to perform due mostly to the difficulties in preparing adequate amounts of structurally intact virus particles which are highly unstable in most density gradient media (4, 1980 annual report).

Three independent observations have recently been made which appear to have largely resolved these problems, at least with respect to these experiments 1) by thin section analysis it was noted that large numbers of virus particles, rather than being released into media supernatants, accumulate within intracellular spaces, often in such concentrations that paracrystalline arrays were formed (shown below). Such particles are, however, released without a substantial increase in host cell contamination if infected cells are washed in TNE Buffer (0.01 M Tris-HCl, pH 7.2, 0.1 M NaCl and 0.002 M EDTA). 2) Although sandfly viruses seem highly unstable in

sucrose, tartrate, glycerol-tartrate, or Percoll (Pharmacia) gradients, they do appear relatively stable in equilibrium gradients composed of Renografin-76 (Squibb) in TNE buffer (15-45% w/w). 3) Negative staining performed with phosphotunstic acid, at any concentration or pH tested, results in positive staining of virus particles with resulting poor resolution. Ammonium molybdate, however, when used as a density contrast medium, results in good preservation with delineation of some envelope spike structure. Therefore, these procedures have been used to examine the morphology of Karimabad virus and in surface iodination reactions catalyzed by Iodogen.

Figure 1 and Figure 2 show the fine structure of Karimabad virus, as seen by ammonium molybdate negative staining of Renografin-purified virus (Figure 1 x 170,000) and of a paracrystalline, intercellular array as monitored by thin section (Figure 2, x 105,000). Similar analysis of Punta Toro virus has yielded essentially equivalent results. In negative stains, particles were generally spherical with an average diameter of 94 nm. Only a small percentage of disaggregated particles was seen. The envelope spikes are clearly seen as a peripheral fringe of 6-7 nm. When viewed at the virion periphery, spikes appear to be broader at their termini than at their base, often giving the appearance of a halo surrounding each particle. When viewed on end, against the particle itself, the spikes are often seen in quasi-circular arrays surrounding a cavity which is penetrated by the stain. In this regard, they resemble the spike structures of Uukuniemi virus (5), although hexagonal packing is not as clear and the spikes seem relatively shorter. The organization of the two envelope proteins in these spikes is unknown and must await the results of the experiments outlined above. However, inhibitions of virus function (neutralization and hemagglutination) by monoclonal antibodies (Section IV-4) suggests that GP-1 and GP-2 are closely associated, at least at some determinants, and may well be in a complex in the same spike structure.

By thin-sectional analysis (Figure 2), virions often appear oval rather than circular, although this may be due to compression during sectioning. A typical bilayered membrane is seen in most particles except those which have been sectioned tangentially. Mean particle diameters do not differ appreciably from measurements made from negative stains if only particles in which this envelope is clearly delineated are measured. A surface fringe of spike structures is again evident peripheral to the envelope. The electron dense internal components (RNP) of extracellular virions are usually seen to be randomly distributed within the core, which is in marked contrast to that seen during budding at Golgi membranes in which the RNP is tightly associated with the forming viral envelope. It is unknown if this indicates that a reorganization of structural proteins occurs following maturation.

Surface-specific iodination was performed with gradient purified Karimabad virus by adding virus and ^{125}I (as sodium iodide, Amersham) to glass tubes previously surface-plated with Iodogen (Pierce Chemical). Iodogen is a mild oxidant, insoluble in water, which gives results equivalent to lactoperoxidase/peroxide systems, but is more convenient insofar as the reaction can be stopped by simply decanting the reaction tube, additional reagents do not need to be added at regular intervals, and tubes once plated are stable for several months. Intact Karimabad virions, and virions previously disrupted in 0.5% SDS were iodinated in this manner, solubilized, and subjected to polyacrylamide electrophoresis. The expected results were obtained insofar as both the nucleocapsid and glycoproteins were labeled in SDS-solubilized virions, but that only the glycoproteins were labeled in intact virions. In addition, a polypeptide of 125,000 daltons was detected in both samples which was not detected in tritiated samples prior to iodination, and is therefore considered to be a dimer of the envelope glycoprotein caused by the known ability of iodine to cross-link proteins (6) (data not presented).

2) Comparison of Polypeptides Specified by 12 Serologically Distinct Sandfly
Fever Viruses

   a. Comparison of Heterologous Strains

   There are currently 31 serologically distinct viruses which have been
placed in the phlebotomus fever serogroup on the basis of reciprocal HI reactions.
In addition, 3 recent isolates from Italy and Brazil are also being considered for
inclusion within this group. Although it is not our intention to study all of
these viruses, most of our work has been concentrated on only two sandfly viruses,
Punta Toro and Karimabad. Thus, in our analysis of the proteins of these two
viruses, we have been uncertain as to what extent the results and conclusions could
be considered characteristic of the group as a whole and which are unique to these
viruses. Consequently, we have extended our studies to identify the virus-coded
proteins of ten additional sandfly viruses, as indicated in Table 1. There were,
in addition, several other factors which prompted this study. 1) To determine if
unique proteins, or combinations of unique proteins, could be detected in 2 or more
viruses. Such data could provide evidence for genome segment reassortment in
nature, and perhaps gene coding assignments (since at least one and probably all
segments code for more than one protein, see Section IV-5). 2) To determine if
nonstructural proteins also could be detected in other sandfly viruses, and 3) These
data were needed to establish the basic information required in the cross-immunopre-
cipitation studies which also have been initiated to trace cross-reacting determi-
nants to specific viral proteins.

   Arumowot, Rio Grande, Saint Floris, Gabek Forest, and 1-47 viruses, as well
as homologous antisera, were obtained from Dr. C. J. Peters (USAMRIID). Naples,
Sicilian, Itaporanga, Chagres and Candiru viruses and homologous antisera were ob-
tained from Dr. W. Brandt (WRAIR). These viruses include all of the known human
pathogens with the exception of Rift Valley Fever virus, which requires high level
containment. These viruses were passaged at low multiplicities in subconfluent
vero cell cultures and harvested when C.P.E. was advanced (48-72 hours). The anti-
sera were adsorbed at $4^{\circ}C$ with uninfected vero cells. Each strain was then used to
infect vero cultures which were incubated overnight and subsequently labeled with
^{3}H-leucine in leucine-free media from 15 to 20 hours after infection. Lysates of
infected cells were prepared for immunoprecipitation and polyacrylamide gel analysis,
as described in Methods. The results, summarized in Table 1, were collected from a
minimum of three independent analyses for each virus. Each antiserum was also
reacted against uninfected cells to control for nonspecific precipitation. Glyco-
proteins were identified either by the incorporation of 2-^{3}H-mannose, altered
migration in cells inhibited with tunicamycin, or reactivity with monoclonal anti-
bodies (see Section IV-4). The identity of nucleocapsid proteins was assumed from
their very similar migration to the known nucleocapsid protein of Karimabad and
Punta Toro viruses and to the observation that these proteins are invariably made in
molar amounts in excess of other virus-specific proteins. The assignment of poly-
peptides as structural or nonstructural must be considered tentative as not all of
the viruses listed in Table 1 have been isolated by gradient centrifugation or by
direct immunoprecipitation of particles from media supernatants.

   Nonetheless, even with these restrictions, the similarities in the profiles
generated by immunoprecipitation are striking. Clearly, similarities exist between
the number and molecular weights of the major virus polypeptides. The nucleocapsid
proteins varied between 23 and 32,000 daltons, and the glycoproteins from 52 to
66,000 daltons. Despite this similarity, most of the viruses could be distinguished
when run in parallel on discontinuous polyacrylamide slab gels. Only Karimabad and
Chagres viruses generated similar profiles.

Table 1

Apparent Molecular Weights ($X10^{-3}$) of Polypeptides
Specified by Selected Phlebotomus Serogroup Viruses

		Structural		Putative Nonstructural
	Nucleocapsid	Glycoprotein	Other	
Punta Toro	27	66, 56	140	(46?), 31, 25, 22
Karimabad	24	59	140	74, 51, 31, 22*, 17*
Arumowot	23	59, 54		74
Naples	25	59, 50		74
Sicilian	26	59		74, 31
Rio Grande	32	66		
Saint Floris	26	66, 52		74
Itaporanga	24	66		26, 35
Chagres	24	59		31
Candiru	26	63		85, 31
Gabek Forest	24	57		32
I-47	27	59		

* detected only in in vitro translation of viral m RNA.

Of the viruses examined, only Punta Toro, St. Floris, Arumowot and Naples viruses produced two glycoprotein species which were consistently resolved by these techniques. However, as shown in Section IV-3, in the presence of tunicamycin, an inhibitor of asparagine-linked glycosylation, two nonglycosylated proteins are detected in Karimabad virus-infected cells, which are otherwise poorly resolved - presumably due to glycosylation microheterogeneity. Thus, it is likely that each sandfly virus codes for two unique glycoproteins and that some factor in morphogenesis or in virus structure requires that these proteins be of similar molecular weight. The presence of two glycoproteins seems, also, to be a general finding in other groups of bunyaviruses(7).

As has been noted in the previous proposal, the combined molecular weight of the 3 genome segments of the sandfly viruses which have been studied (7) is greatly in excess of that required to code for the major structural proteins and the assumed presence of a virion-associated transcriptase which is mandated by the negative polarity of the genome. Nonstructural proteins, however, have not been reported previously in cells infected with any of the bunyaviruses (4). Although it is possible that bunyaviruses devote a larger proportion of their genome to non-coding sequences, it is more likely that nonstructural proteins exist in low molar concentrations relative to the structural proteins and the techniques which have been used are insufficiently sensitive to detect these polypeptides.

By immunoprecipitation of infected cell lysates using hyperimmune mouse ascitic fluids (prepared by immunization with infected suckling mouse brain), we have detected 3 nonstructural proteins in Karimabad-infected cells of 31,000, 51,000 and 74,000 daltons (reviewed in 1980 Annual Report). We now know that the 51K protein contains tryptic peptides in common with the major virion glycoproteins (Section IV-6), is co-precipitated with these proteins by a cross-reacting Punta Toro virus monoclonal antibody (Section IV-5), and co-migrates with a major virion envelope protein which

is produced in the presence of tunicamycin (Section IV-3). Therefore, the 51K polypeptide is assumed to be an underglycosylated form of one of the Karimabad virus glycoproteins. As indicated in the preceeding Annual Report, the 74K polypeptide has not been found in uninfected cell lysates, but it is present in only low amounts from infected cells, and has not been detected in all of our gels. Pulse-chase experiments, or experiments in which protease inhibitors or amino acid analogues have been used, have failed to significantly alter the migration or the relative amount of this protein. However, if virus is subjected to varying doses of ultraviolet light before being used for infection, the synthesis of this protein is reduced in a dose-dependent fashion. In addition, it is detected in both BHK and vero cells. Therefore, this protein is either a nonstructural protein synthesized in low quantities, (or alternatively is inefficiently precipitated) or a host cell-specific polypeptide which is induced following infection and adheres to our immunoprecipitates.

By contrast, the 31K protein is detected in large amounts in cells infected with Karimabad virus and in in vitro translations of viral messenger RNA, and has been studied in greater detail. The results of these experiments are presented in Section IV-5.

Therefore, in light of our studies on the putative nonstructural proteins of Karimabad virus, it is of interest that 5 of the 12 sandfly viruses which we have examined, code for a protein which comigrates with the 31K polypeptide of Karimabad virus, that Gabek Forest virus and Itaporanga virus produce a 32K and 35K polypeptide respectively, and that such a protein would probably not be detected in Rio Grande virus due to the unusually high molecular weight of the nucleocapsid protein. Similarly, it is of interest that a 74K protein is detected in cells infected with at least 5 of these viruses. In summary, it would appear that there are broad similarities among the structural proteins and probably also among nonstructural proteins of the various viruses of the sandfly group. This would suggest that, at least with respect to these proteins and the mechanisms which control their synthesis, conclusions reached from the study of representative strains can be safely extrapolated to other members of the group. Unfortunately, we have not yet found a sandfly virus which both produces large amounts of the 31K nonstructural protein (as does Karimabad) and also codes for glycoproteins which readily separate by one-dimensional electrophoresis (as does Punta Toro). Consequently, we will continue to study both of these viruses to take advantage of these characteristics, as is indicated in the following sections.

The analys'   of these additional strains of sandfly viruses has yielded one other finding th-   ᶜ direct interest to our studies which have sought to determine the manner .   ᵓxpression of sandfly virus genomes are controlled, that is whether monoc'   ᶜ   polycistronic messengers are produced. Considering that eukaryotic cᵤ   pparently incapable of internal initiation for translation on polycistronic m   ᵍᵢ rs, such messengers must translate a precursor protein which is subsequently cleaved to yield mature proteins. In cells infected with Candiru virus, a protein of 85,000 daltons has been consistently immunoprecipitated and is present in higher relative amounts in short pulse-labeling experiments. This polypeptide co-migrates with a polypeptide produced in Punta Toro virus-infected cells which have been treated with amino acid analogues (1980 Annual Report). These data are consistent with the interpretation that this protein is a precursor, and that its cleavage occurs at lower rates in Candiru virus-infected cells. Some of the monoclonal antibodies produced against Punta Toro virus antigens react with the glycoproteins of Candiru virus and others against the Candiru virus nucleoprotein. These reagents are currently being used to determine if the 85,000 dalton protein is coprecipitated with other structural or nonstructural proteins.

b. Comparison of the Polypeptides of Six Strains of Punta Toro Virus
    Which Differ in Virulence for Laboratory Animals

Since its original isolation from acute phase serum from an individual
exposed in Panama, several isolations of Punta Toro · virus have been made from
various geographic locations in Panama. In one study on the ecology of sandfly
viruses in Panama, Tesh reported that fifty separate isolations of Punta Toro virus
were made in vero cells from pools of sandflies (Lutzomyia species). Tesh's work
also demonstrated that, based on the presence of neutralizing antibodies, a signi-
ficant incidence of human infection (6-40%) existed among inhabitants of several
rural villages (8). This work was conducted in the Canal Zone proper or in western
Panama. Since the completion of these studies, 3 isolations of Punta Toro virus
have been made in eastern Panama. In an analysis of these 3 strains, Peters found
that each was invariably fatal when inoculated into hamsters,whereas strains from
western Panama produced no or few deaths (9). A similar difference in virulence
(after peripheral challenge) has also been noted with some of these strains in mice
(see renewal application).

As is discussed below and in the accompanying application, we have
initiated experiments in mice which will seek to define the biological role of
individual Punta Toro virus glycoproteins and specific antigenic determinants in
providing immunity to viral challenge. These studies are being approached by passive
immunization with monoclonal antibodies and polyclonal antisera, and by active immu-
nization with affinity-purified viral proteins. Therefore, it was of interest to
compare the polypeptides of these strains of Punta Toro virus to determine if dif-
ferences in virulence could be correlated with specific changes in polypeptide
profiles generated by immunoprecipitation.

These strains were obtained from Dr. C. J. Peters (USAMRIID) and were
not passaged prior to their analysis. Each was inoculated into vero cultures which
were labeled with ^{3}H-leucine, and immunoprecipitation and polyacrylamide gel analysis
were carried out as described in Methods. The results of this analysis are presented
in Figure 3. The virulent strains (from eastern Panama) are labeled 4-6, and the
strains with decreased virulence for laboratory animals are labeled 1-3. As can
be seen from these data, the polypeptide profiles generated from all of these strains
are nearly identical. No differences are noted in the migration of the nucleocapsid
proteins or the two nonstructural polypeptides which migrate faster than the nucleo-
capsid protein. Minor (but reproducible) differences in the mobilities of the two
glycoproteins are noted in which those from two of the less virulent strains show a
slightly broader separation than those of the 3 virulent strains (all of which appear
identical). The avirulent strain (labeled #3), however, is not differentiated from
the 3 virulent strains. Such minor differences in migration could be related to
glycosylation microheterogeneity or to a slightly different cleavage site of a pre-
cursor polypeptide. In summary, this analysis has not shown major differences among
the polypeptides specified by strains of varying virulence which are detectable by
molecular weight analysis. However, it is clear that some variation in the envelope
proteins of these viruses exists.

Experiments are proposed in the accompanying application which will
further examine the characteristics of these viruses both in vivo and in vitro in
an attempt to determine the basis for the significant differences which exist in
their relative virulence.

3) <u>The Effect of Tunicamycin on the Synthesis of Sandfly Fever Virus
Envelope Proteins (Punta Toro and Karimabad Viruses)</u>

As indicated in the previous report, we have analyzed the glycopeptides
of Karimabad and Punta Toro virus glycoproteins based on the immunoprecipitation
of 2-^{3}H-mannose-labeled proteins from lysates of infected cells. The results from
these two viruses were similar. The conclusions from these studies are:

1. that virus glycoproteins are glycosylated exclusively at asparagine
residues (based on mild base stability studies and tunicamycin
sensitivity);

2. that only polymannose (simple) type oligosaccharides are present as
determined by Bio-Gel P-6 chromatography and α-mannosidase sensitivity.
Consequently, only N-acetylglucosamine and mannose residues are used
to construct these oligosaccharides.

3. that the size of the oligosaccharide unit (based on Bio-Gel P-6
chromatography) is sufficient to contain only 5-6 mannose residues
linked to the two N-acetylglucosamine residues;

4. that as no separation of the two glycoproteins was made, and only
one type of glycopeptide was found, that similar oligosaccharides
are present on both glycoproteins.

One aspect of these results was not expected, specifically, that no complex
oligosaccharide chains were detected. In all other enveloped viruses which have
been studied, either only complex or combinations of complex and simple oligosaccharide
chains are found. One possible explanation for the unique situation observed in sand-
fly virus glycopeptides is that these viruses modify the Golgi membranes with viral
glycoproteins in the process of budding and maturation, which is known to occur at
these membranes from electron microscopy studies. Since the normal cell glycosyl-
transferases which construct the complex chains are also Golgi membrane proteins,
these enzymes may be removed or rendered inactive by the viral maturation process.

However, we could not exclude the possibility that most of the viral glyco-
proteins, present in the cell at a given time, were immature in the sense that Golgi
modification had not yet occurred. This was considered unlikely as due to the internal
maturation of these viruses, substantial numbers of viral particles exist intracellu-
larly at the time of harvest, and large numbers of virus particles also adhere to the
cell periphery (Section IV-1), all of which should have been collected during the lysis
and immunoprecipitation procedures. Nonetheless, rigorous demonstration that mature
glycoproteins have, in fact, only polymannose oligosaccharides must depend on analysis
of released and purified virus particles. Thus, the conclusion of these studies has
been hampered by the inability to obtain sufficient quantities of 2-^{3}H-mannose-labeled
purified virions. On the basis of the results presented above, in which virus particles
appear stable in Renografin-76 gradients, these studies can now be concluded.

The studies summarized above do, however, show that all oligosaccharides
are asparagine-linked. Therefore, tunicamycin, which inhibits the glycosylation of
the lipid intermediates responsible for the transfer of the preformed oligosaccharides
to asparagine residues (10) should abolish glycosylation of sandfly virus envelope
proteins, if present in sufficient concentration. The effect of this inhibitor on

the migration of Punta Toro and Karimabad virus glycoproteins in polyacrylamide gels has been determined. The information which was expected to be gained from these experiments is the following: 1) The molecular weights of the nascent, unglycosylated polypeptides. This information is required to determine the migration expected of the unglycosylated polypeptides which could be produced in in vitro translation, or the sum of these polypeptides if they are synthesized as an unglycosylated precursor, either in infected cells or in in vitro translations (Section IV-5). 2) The minimum number of oligosaccharide units transferred to the nascent envelope polypeptides, based on the number of intermediate bands produced. 3) Whether the presence of tunicamycin inhibits the cleavage of precursor polypeptides, should they exist, as has been shown for nonglycosylated precursors of other viruses (11, 12).

The effect of tunicamycin on the incorporation of ^{3}H-leucine and 2-^{3}H-mannose into immunoprecipitated Karimabad and Punta Toro viral proteins is shown in Table 2.

Table 2

Inhibition of Glycosylation of Viral Glycoproteins by Tunicamycin[1]

Tunicamycin Concentration (µg/ml)	Incorporation of ^{3}H-leucine		Incorporation[2] of 2-^{3}H-mannose	
	PTV	KAR	PTV	KAR
0	4.0×10^5	1.7×10^6	3.7×10^4	6.3×10^5
1	3.2×10^5 (80%)	1.3×10^6 (76%)	1.0×10^4 (27%)	2.6×10^5 (41%)
5	2.5×10^5 (62%)	1.0×10^6 (58%)	0.3×10^4 (8.0%)	0.1×10^5 (2.2%)

[1] Tunicamycin added 4 hours before addition of labeled precursor
[2] Incorporation of the indicated precursor into immunoprecipitated viral proteins using homologous hyperimmune ascitic fluids

The data presented in this table shows that tunicamycin, when added at four hours prior to labeling and at 5 µg/ml, inhibits glycosylation of Karimabad and Punta Toro virus proteins at a level exceeding 90% while the effect on overall protein synthesis is much less drastic. If labeling is initiated at one hour after the addition of tunicamycin, or if the inhibitor is present in lower concentrations, a lessened effect is noted which is also indicated by gel analysis (see below.). Thus, the inhibitory effect of tunicamycin is both time and dose dependent. The polyacrylamide gel profile of ^{3}H-leucine-labeled proteins immunoprecipitated from Punta Toro virus-infected cells treated with tunicamycin (at 0, 0.5, 1, 2, and 5 µg/ml), and for 1 hour or 4 hours prior to the addition of ^{3}H-leucine, is shown in Figure 4. The results from similarly treated, but uninfected cells, are included for comparison. Equivalent amounts of radioactivity were loaded onto the gel for each sample so that qualitative differences would be apparent. As can be seen from this figure, the migration of the nucleocapsid protein and that of a nonstructural

25K protein which is known not to be glycosylated (but whose function is otherwise unknown),is not affected by the presence of tunicamycin. In contrast, the migrations of the 2 Punta Toro virus glycoproteins increase in the presence of tunicamycin both as a function of inhibitor concentration and time of treatment. The migration of GP-1 increases in one increment equivalent to a loss of about 3,000 daltons and then remains constant as the concentration of tunicamycin is increased. The migration of GP-2, however, increases in 2 increments of about 2,000 daltons each and then remains constant in higher concentrations of tunicamycin. Similar partial effects have been seen in other virus systems (13). These data suggest 1) that the 63K and 52K polypeptides are the unglycosylated forms of GP-1 and GP-2, respectively, and 2) that GP-1 has fewer sites for glycosylation than GP-2, perhaps only 1 and 2, respectively.

In experiments run in parallel with those reported above, polyacrylamide gel profiles have been produced with $2-^3H$-mannose-labeled proteins and have shown that in the presence of 1 μg of tunicamycin per ml, and with a four hour pretreatment, only the 66K GP-1 polypeptide and the 56K and 54K GP-2 are labeled (data not shown). Therefore, the 63K and the 52K polypeptides, in fact, represent the molecular weight of the unglycosylated envelope proteins.

Similar experiments have been performed with Karimabad virus and the results are shown in Figure 5. In the presence of tunicamycin, the unglycosylated nucleocapsid and 31K nonstructural proteins are not altered in their migration. The Karimabad virus glycoproteins, however, which normally are not resolved by one-dimensional gel analysis, do resolve into two discrete bands of increased mobility when produced in cells inhibited with tunicamycin (either 1 or 5 μg/ml). These bands are not detected in uninfected cells (labeled "uc" on Figure 5) and do not label with $2-^3H$-mannose. Intermediate bands, as seen with Punta Toro virus glycoproteins are not seen. However, the increase in migration of one of these polypeptides appears greater than the other. This suggests that one is normally more heavily glycosylated than the other, as in the case with Punta Toro glycoproteins. The apparent molecular weights of the two unglycosylated Karimabad envelope proteins are 52,000 and 58,000 daltons. It is likley that these methods would also be useful in the resolution of envelope proteins from other sandfly fever viruses, which do not separate by standard one-dimensional polyacrylamide gel analysis.

Polypeptides of higher molecular weight and precipitable with homologous antisera were not apparent in these experiments in which tunicamycin was used to inhibit glycosylation. Therefore, if the glycoproteins are produced from a precursor (polycistronic transcription), its cleavage would appear uneffected by the degree of glycosylation. The cleavage of the Sindbis virus precursor, PE_2, which is inhibited in the absence of glycosylation, may be indirectly affected insofar as its cleavage appears to occur at the cell surface (14), and the migration to the plasma membrane of this and other viral membrane proteins is impaired if they are not glycosylated (11).

If an unglycosylated precursor exists for the two glycoproteins of sandfly fever viruses (as has been shown for the segmented, negative-stranded arenaviruses, (15)),and contains no other amino acid sequences, its molecular weight would be approximately 110,000 daltons for Karimabad virus and 115,000 daltons for Punta Toro virus. Such polypeptides are normally not detected in either steady-state or pulse-labeled cultures infected with either of these viruses. However, in the presence of zinc ions (0.5 mM), a protease inhibitor which has been shown to inhibit the cleavage of several viral precursor proteins (16,17), small amounts of polypeptides

with molecular weights within experimental error of these figures are detected by
immunoprecipitation (1980 Annual Report and Section IV-5). Therefore, it is
possible that such precursors are made but are normally cleaved rapidly, perhaps
as cotranslational events. A high molecular weight polypeptide has also been
translated in vitro from isolated mRNA (Section IV-5). Although we have not yet
obtained sufficient quantities of these zinc-inhibited polypeptides to enable
tryptic peptide analysis, as was done with the 31K nonstructural Karimabad poly-
peptide (shown below), it is expected that the monoclonal antibodies described
below and in Section IV-4, will be useful in determining if these putative pre-
cursors contain determinants in common with known virus-coded proteins.

Four monoclonal antibodies specific for GP-2 and one specific for GP-1
have been isolated. In recent experiments we have shown that all four GP-2 specific
monoclones immunoprecipitate the three GP-2-related polypeptides produced in cells
treated with tunicamycin, and thus the degree of glycosylation does not appear to
effect their binding. However, in two experiments we have not been able to effi-
ciently precipitate the unglycosylated GP-1 polypeptide with the monoclone which
reacts strongly with this polypeptide in its glycosylated form. Therefore, based
on these preliminary results, it would appear that either the carbohydrate moiety
forms part of the antigenic determinant recognized by this specific antibody (as
has recently been seen in other systems (12)), or that in the absence of glycosyla-
tion the conformation of the protein is altered such that the determinant normally
bound by this monoclone is unavailable. This is perhaps not a trivial point, since
this monoclonal antibody has a high neutralization index, and is capable of
affording protection when used in passive immunization in mice inoculated with an
otherwise lethal dose of Punta Toro virus (Section IV-6). In the design of experi-
mental vaccines, especially those produced in prokaryotic systems (as has been
suggested for Rift Valley Fever virus) in which glycosylation does not occur, it
may be important to determine if glycosylation is required for proper protein con-
formation or otherwise affects its immunogenicity relative to the antigen present
on the virulent virus.

4) Monoclonal Antibodies to Punta Toro Virus Antigens

A very substantial effort in the current contract year has been to charac-
terize a panel of monoclonal antibodies directed against the antigens of Punta Toro
virus. These studies have been done in collaboration with Dr. J. Dalrymple and M.
K. Gentry (WRAIR). Immunizations, initial screening, and cloning were performed at
WRAIR and the specificity of each hybridoma with respect to its target protein were
determined on individual media supernatants at the University of Maryland, by immu-
noprecipitation and acrylamide gel analysis. One hundred and ninety-three positive
clones were examined in this manner. As described in the preceeding application and
summarized below, this effort was considered worthwhile because of the large amount
of information which could be expected both from in vivo and in vitro experiments
with such high titered reagents of unique specificity.

Specifically, it was hoped that these reagents would be useful:

1. to determine which viral proteins contain the determinants responsible
   for neutralization and hemagglutination;

2. to determine the antigenic relationships shared between heterologous
   viruses, and between various structural and nonstructrual proteins of
   individual viruses;

3. to indirectly determine the expected role of the target proteins
    in inducing protective immunity based on the protective effect
    afforded by passive immunization;

4. in the preparation of relatively large amounts of purified viral
    antigens based on immuno-affinity techniques; and

5. in the preparation of highly specific diagnostic reagents.

Punta Toro virus was selected for these studies because 1) it is a known
human pathogen, 2) it is known to cross-react with Rift Valley Fever virus, as well
as several other sandfly viruses, based on reciprocal HI, neutralization, and
immunoprecipitation reactions, 3) the glycoproteins are readily separated by stan-
dard one-dimensional SDS-polyacrylamide gel procedures, 4) this virus is currently
being analyzed in vitro, to study the replication and morphogenesis of sandfly viruses,
and in vivo, to determine which antigens and antigenic determinants are relevant to
the induction of immunity, and 5) strains of Punta Toro virus which differ in their
virulence for hamsters and mice have been isolated, and thus the existence of a panel
of monoclonal antibodies could be useful in correlating virulence to specific viral
polypeptides or antigenic determinants.

Balb/c mice were immunized with suspensions of suckling mouse brain which
should contain all structural and nonstructural proteins of Punta Toro virus with
a minimum of foreign host cell determinants. A secondary response was initiated by
booster immunization with the same material at eight weeks (i.v.). Three days after
the second immunization, spleen cells were harvested, washed, and fused with the
P3/X63-Ag8 myeloma line (Balb/c) derived from MOP C-21 by C. Milstein (19). Fusion
was accomplished by polyethylene glycol and hybrids were cloned in 96 well plates
by the methods described by Kennet, et al. (20). Media supernatants from actively
growing clones were screened by solid phase RIA using Punta Toro virus-infected cells
lysed with nonionic detergents as antigen. Actively growing hybridomas (in HAT selec-
tive media) were produced in 675 wells of which 435, or 64%, were RIA positive.
Approximately one-third of these media supernatants were also screened at the
University of Maryland by indirect fluorescent antibody (FA) procedures using cells
growing on teflon-coated spot slides and fixed with acetone at 16 hours after infec-
tion. The FA analysis was performed to enable selection of any clones exihibiting a
unique distribution of antigens, and to determine the efficiency of this technique
relative to RIA in detecting antibody-positive wells. Although a good correlation
was obtained between FA and RIA, indicating that FA was sufficiently sensitive to
be used as a screening technique, dramatic differences in intracellular antigen
distributions were not detected. This, however, is not surprising considering, as
described below, that most antibody-positive hybridomas secreted immunoglobulins
reactive to the nucleocapsid protein.

Of the 435 positive cultures, 105 were cloned in soft agar over a feeder
layer of human fibroblasts. Representative subclones were then chosen from each of
99 cultures, reexamined for RIA reactivity, and on this basis one to three subclones
were selected from each culture (N=193) for further study. These subclones were
replicated in 75 cm^2 flasks, the supernatant media saved, and the cells frozen in
liquid nitrogen. The monoclonal antibodies secreted into the media of the 75 cm^2
flasks were then analyzed to determine the viral protein carrying the target anti-
genic determinant.

Vero cells were infected with Punta Toro virus and labeled with 100 μCi/ml of
^{3}H-leucine from 16-20 hours after infection in media otherwise free of leucine.
The cultures were then lysed in a nonionic detergent-containing buffer (as described
in Methods) and aliquots of this lysate were reacted with 50 μl of each hybridoma
supernatant medium overnight at 4°C. Immune complexes were then adsorbed onto
glutaraldehyde-fixed staphylococcal A cells, washed and prepared for SDS polyacryla-
mide gel electrophoresis and fluorography,as described in Methods. Immunoprecipita-
tion with Punta Toro HMAF served to standardize the gel and mark the migration of
viral polypeptides. A composite gel demonstrating the utility of this method and
hybridomas reactive to GP-1, GP-2 or nucleocapsid protein is shown in Figure 6.

As shown in Table 3, of the 193 subclones selected, 168 reacted with the
nucleocapsid protein on initial immunoprecipitations or subsequent immunoprecipita-
tions with labeled lysates of higher specific activities. When a mixture of rabbit
anti-mouse IgA, IgM, and IgG$_1$ (Bionetics, Kensington, MD) was added to the reaction
mixture, an additional 7 subclones were shown to be reactive to nucleocapsid protein
by subsequent gel analysis, indicating that these clones secreted an antibody class
or subclass not reactive with protein A. Seven of the subclones, representing 4
separate parent clones, reacted with GP-2. Only two subclones, both from the same
parental clone, reacted with GP-1. Nine of the subclones were unreactive by these
procedures, indicating that antibodies were no longer secreted or that they were
present in insufficient concentration. Thus, of the 99 original parental clones,
93 produced nucleocapsid antibody, from one or more subclones (usually all), 4 pro-
duced anti-GP-2, one produced anti-GP-1, and one was undetermined. In no case did
subclones from a single parental clone react with different viral proteins.

Table 3

Selection and Antigen Specificities of Punta Toro Virus (PTV) Monoclonal Antibodies

	Frequency
Hybridoma Positive Cultures	675/864
Positive for PTV Antibodies	435/675
Selected for Further Study	105/435
Analyzed by Immunoprecipitation (193 subclones)	99/105
Positive for PTV nucleocapsid Protein (Protein A Reactive)	89/99
Positive for PTV nucleocapsid (Protein A Non-reactive)	4/99
Positive for GP-2	4/99
Positive for GP-1	1/99
Undetermined	1/99

As the concentrations of viral proteins present in these brain homogenates
which were used for immunization is unknown, as is their relative immunogenicity, it
was considered likely that an unequal distribution of specificities could be obtained,
despite the fact that HMAF produced by similar immunizations contains strong activi-
ties against all three proteins. Thus, fluorescent antibody analyses were performed,
and clones with widely divergent RIA titers were selected, in an attempt to select

clones with as many different specificities as possible. However, such a strong
bias in favor of the nucleocapsid protein was not expected. In fact, all of the
glycoprotein-reacting clones generated low RIA titers, and would have been dis-
carded if some such low-titered clones were not intentionally selected. It is not
known whether the bias in favor of the nucleocapsid protein is a function of its
relative concentration, its relative immunogenicity, or its relative ability to
bind to the (polyvinyl chloride) RIA plates. However, it is certainly true that
the nucleocapsid protein is produced in amounts greatly in excess of the glyco-
proteins in cells infected in vitro, and can easily be detected by coomassie blue
staining of gels over an unfractionated host cell background. Therefore, although
multiple explanations exist with which to explain our results, it is nonetheless
unfortunate that more monoclones were not selected which are reactive to Punta Toro
virus glycoproteins, polymerases, or other intracellular proteins, as this decreases
the number of studies which can be conducted with this or heterologous sandfly
viruses. For example, none of the glycoprotein-reacting clones neutralizes, or
inhibits the hemagglutination, induced by Rift Valley Fever virus (9)

Subsequent fusions are planned (in continuing collaboration with Dr. J.
Dalrymple) in which conditions will be altered to favor the selection of glyco-
protein-reactive hybridomas. However, the existing monoclones have provided, and
are expected to continue to provide, very useful information, as indicated below:

1. They facilitate studies designed to assay the interrelationships
   between Punta Toro virus specific proteins, such as precursor-
   product relationships, identity of unglycosylated membrane proteins
   (Section IV-3), or identity of peptides produced in in vitro transla-
   tion assays (Section IV-5).

2. As described in Section IV-6, the monoclone reactive to GP-1, which
   will be referred to as 7F5-7 (GP-1), is capable of providing complete
   protection to animals when passively transferred (i.p.) before and
   after virus inoculation. Such protection is not conferred by trans-
   fer of GP-2 reacting monoclones (at least in mice), indicating that
   such studies may identify the antigens or antigenic determinants
   relevant to providing immunologic protection, and hence relevant
   to the design of vaccines.

3. Although these monoclones have not been reacted to many other hetero-
   logous sandfly viruses, one of the GP-2 specific monoclones, 3A8-7
   (GP-2) reacts with a Karimabad glycoprotein and 3A8-7 (GP-2), 9G6-7
   (GP-2), and 4A9-6 (GP-2) react against Candiru virus glycoproteins
   (Table 4). 7F5-7 (GP-1) has so far only been shown to react
   with Punta Toro virus.

Table 4

Characteristics of Selected Monoclonal Antibodies[1] to Punta Toro Virus Proteins

Clone and Specificity	HI Titer	Neut. Titer[2]	Reactivity to proteins of:		Passive Protection (mice)[5]
			Karimabad	Candiru	
7F5-7 (GP-1)	320	6,300	-	-	+
3A8-7 (GP-2)	640	2	+(GP)	+(GP)	-
9G6-7 (GP-2)	1280	2,000	-	+(GP)	-
4G2-6 (GP-2)	160	2	-	-	-
4A9-6 (GP-2)	320	24	-	+(GP)	-
1A10-1 (NC[3])	<10	<2	-	+	-
8B6-6 (NC)	<10	<2	+(NC)	+	NT[4]
HMAF	320	13,600	+	+	+

1. From ascitic fluids produced in Balb/c, pristane-primed mice.
2. Determined at USAMRIID by Drs. Dalrymple and Peters
3. NC = nucleocapsid protein.
4. NT = not tested   5. See table 9

These results are of interest from several points of view. First, the two glycoproteins of both Karimabad and Candiru viruses are of similar molecular weights and do not separate by SDS gel electrophoresis. Thus, these mono-clones will be useful in the separation of these glycoproteins both analytically and preparatively. Second, this indicates that specific diagnostic reagents may be prepared by this technique. Third, such heterologous reactions are useful in determining whether monoclones reactive to a given viral protein identify the same or different antigenic determinants as described below. Fourth, the monoclones may also be used in assessing the interrelationships of proteins of these heterologous viruses, as suggested above for Punta Toro virus. Of particular interest is the relationship between the Karimabad virus nucleocapsid and 31K nonstructural protein (see Section IV-5).

4. One of the objectives of this study was to correlate the neutralization and HI activities of the various monoclonal antibodies with the target antigens as determined by immunoprecipitation. In this manner, it was hoped to determine which glycoprotein contained the antigenic determinants responsible for these serological reactions. Precedent exists for having these activities on a single glycoprotein (flaviviruses), on one of two glycoproteins (influenza, parainfluenza) or on two separate glycoproteins (Sindbis). As shown in Table 4, three of the GP-2 specific monoclones exhibited HI activities without significant neutralization activities. However, one monoclone shown by immunoprecipitation to react only with GP-2 and another which reacted only with GP-1, demonstrated both HI and neutralization capacities. Nonetheless, the relative HI and neutralization titers differed such that the GP-2 reactor demonstrated a high HI titer relative to its neutralization titer, and the GP-1 reactor gave the reciprocal results. Although other interpretations are possible, the most straightforward explanation of these data is that the major determinant monitored by neutralization resides on

GP-1, and the major determinant which is monitored by HI resides on
GP-2, but that these determinants are juxtaposed sufficiently closely
in the mature virion that antibody binding to one determinant sterically
inhibits the other. Consequently, it is likely that the envelope spikes
are composed of some multiple of GP-1 and GP-2, as has been proposed
for alphaviruses (21), rather than being composed of only one of these
proteins, as has been shown for influenza viruses (22).

The hypothesis that places the major HI determinant on GP-2 is also streng-
thened by the indication that the four GP-2 specific monoclones probably detect
different determinants. This is based on the data from experiments in which the
reactivities of these monoclones to Karimabad and Candiru virus proteins were
determined by immunoprecipitation (Table 4). These differential reactivities
combined with the observation that only 9G6-7 (GP-2) is capable of neutralization
suggest that each monoclone recognizes a distinct determinant. Consequently,
the binding of antibody to 4 different sites on GP-2 inhibits hemagglutination
to some degree, perhaps due in part to induced conformational changes, but anti-
body binding to only one of these sites is capable of neutralization.

5) Control of the Expression of Sandfly Fever Virus Genomes and Interrela-
     tionships of Currently Recognized Proteins

One of the major objectives of our studies which have characterized the
structural and nonstructural proteins of sandfly fever viruses has been to deter-
mine the manner in which the expression of the genomes of these viruses is con-
trolled and the interrelationships of the viral proteins which have been identified.
The central observations are clear: 1) Sandfly viruses specify four structural
proteins (two glycoproteins, a nucleocapsid protein, and a virion-associated poly-
merase), at least one major nonstructural protein (31K) and perhaps several others,
as indicated above, and 2) they produce these proteins from only three negative-
stranded genome segments, each of which contains unique nucleotide sequences as
monitored by two-dimensional oligonucleotide mapping techniques (4). It will
be shown below that the 31K nonstructural protein of Karimabad virus contains, for
the most part, unique tryptic peptides when compared to the other major viral
polypeptides. Thus, the number of virus-specific proteins exceeds the number of
genome segments by at least two, and probably more, since the theoretical coding
capability of these viruses is in excess of that which would be required to code
for the currently recognized proteins. Consequently, at least some segments must
produce either multiple monocistronic messenger RNA's, or alternatively polycis-
tronic messengers, as there appears to be no internal initiation for translation
on eukaryotic mRNA's.

Among negative-stranded RNA viruses, precedent exists for both monocis-
tronic transcription (e.g., rhabdoviruses, paramyxoviruses and myxoviruses) and
polycistronic transcription (arenaviruses). With the recent demonstration that
messenger splicing occurs during mRNA synthesis from at least one segment of
influenza viruses (23), variations on these two alternative schemes are at least
theoretically possible for bunyaviruses. Thus, non-adjacent nucleotide sequences
may be ligated either from the same or different reading frames to form unique
mRNA's. (This possibility will be discussed below in reference to recent data
that suggests that the 31K and nucleocapsid proteins of Karimabad virus may share
some tryptic peptides.) But, however produced, the existence of polycistronic
mRNA's assumes the synthesis of precursor proteins which subsequently must be
cleaved for the formation of mature viral proteins. Thus, if precursor proteins

could be demonstrated in sandfly fever virus-infected cells, the corresponding viral genes must be transcribed polycistronically. Furthermore, the study of such precursors could indicate the location of these genes relative to each other, and a comparison of messenger and genome segment molecular weights could suggest possible coding assignments of the individual genomic segments.

Therefore, making the assumption that the polyclonal antisera used in our immunoprecipitation assays would recognize such precursors, several experiments have sought to demonstrate their presence in sandfly fever virus infected cells focusing, for the most part, on Karimabad and Punta Toro viruses (previous annual report). Most of these experiments have resulted in negative findings. Pulse-chase studies, in which all of the viral proteins described in Section IV-2 could be identified with labeling times as short as five mintues, failed to reveal high molecular weight polypeptides, and all polypeptides were metabolically stable. Similarly, the incorporation of amino acid analogues (fluorophenylalanine, azetidine, ethionine, and canavanine) in concentrations which have been shown to result in the accumulation of uncleavable precursors in other virus systems (16,24), failed to provide definitive evidence for high molecular weight precursors, in either Karimabad or Punta Toro virus-infected cells.

These data, however, cannot be construed as evidence that such precursor polypeptides are not synthesized as the chances for success of pulse-chase procedures are a function of the time interval between translation and cleavage, and negative data from the use of amino acid analogues cannot be interpreted. For example, the relationship between the PE_2 and E_2 proteins of Sindbis virus s easily demonstrable because the cleavage of PE_2 is delayed until final maturation of the virion (14). On the other hand, the cleavage of the 130 and 100K precursor polypeptides are cotranslational events which are not demonstrable by pulse-chase methodology, and have been shown only by an analysis of temperature-sensitive mutants or in the presence of proteolytic inhibitors (14, 17, 25).

Therefore, in current studies which have sought to identify precursor molecules and to identify interrelationships among currently recognized polypeptides, four different experimental approaches have been taken and are being pursued concurrently. 1) Transcriptional mapping by U.V. irradiation, 2) tryptic peptide analysis of Karimabad structural and nonstructural proteins, 3) inhibition proteolysis by zinc ions, and 4) in vitro translation of Karimabad virus mRNA.

a. Transcriptional mapping by U.V. irradiation

Using modification of the procedures developed by Saurbier (26) U.V. transcriptional mapping procedures have been used to study transcriptional controls of several RNA viruses (27,28). The essential feature of this technique, as described in the preceeding application, is that a U.V. dimer produced in the template strand, prevents transcription of mRNA past that point. Consequently, if all viral genes possess independent promoters and are transcribed independently, the inactivation of mRNA synthesis will be directly proportional to cistron molecular weight. On the other hand, if the number of promoters is less than the number of genes, the transcription of a gene distant from the promoter will be more sensitive than those which are promoter proximal, and inactivated as the sum of its own molecular weight plus the size of the intervening genes. Therefore, mature polypeptides translated from polycistronic messengers would be inactivated coordinantly, whereas those produced from monocistronic messengers from promoter proximal genes would be inactivated as a direct function of their individual molecular weights (target size).

An additional possibility, however, is reflected in the results obtained with VSV. In this system the synthesis of individual proteins following U.V. irradiation was shown to be coordinant despite the fact that monocistronic messengers are known to be synthesized. These results were used to demonstrate that the entire VSV genome behaved as a single transcriptional unit and that each gene was transcribed in turn from a single promoter, at or near the 3' end (27). Therefore, this technique adequately differentiates single from multiple promoter models, but could not distinguish a situation in which polycistronic messengers were produced, from one similar to that seen in VSV, without additional information.

The use of this technique in bunyaviruses is further complicated by the existence of the segmented genome, however, it was expected that multiple or single promoter models could be documented, and in the later event, differentiate those genes which were promoter proximal from those promoter distal.

Therefore, Karimabad and Punta Toro viruses were concentrated from stocks by centrifugation and resuspended in 5 mls of Hanks' balanced salt solution lacking phenol red but containing 2% fetal calf serum. These virus suspensions were then given graded doses of ultraviolet light equivalent to 50-300 ergs/mm^2 (greater than 95% inactivation), and used to infect vero cell cultures at low multiplicities (based on the titer of unirradiated virus) to avoid multiplicity reactivation. ^{3}H-leucine was then added to these cultures at various times after infection and the proteins produced were immunoprecipitated and analyzed by gel electrophoresis. The results of these studies were disappointing. Although it could be shown that the synthesis of viral proteins was decreased as a function of their molecular weight, including the two Punta Toro virus glycoproteins, insufficient incorporation of label was attained unless cultures were incubated for 12-14 hours. Therefore, during this time, undamaged genome segments would be expected to replicate at unknown rates as would residual infectious virus. Since these procedures depend on the monitoring of primary transcription ((27) and preceeding application), the data from these experiments cannot be interpreted with confidence.

Similar experiments are possible, however, based on the recent findings of Fuller and Marcus (29), who have demonstrated that translation as well as transcription cannot proceed past a UV-induced dimer. Thus, cells in advanced stages of infection, or extracted mRNA to be translated directly in in vitro translation systems, (Section IV-5) can be irradiated, and the effect of irradiation monitored by direct protein gel analysis. These procedures are thus 1) more convenient, 2) can be initiated during times of maximal viral protein synthesis, and 3) allow some conclusions to be drawn with respect to the existence of monocistronic or polycistronic messengers since the effect of UV irradiation can be monitored directly. Such experiments are proposed in the accompanying application to be run in parallel with expanded in vitro translation experiments, gradient analysis of mRNA's, and pactamycin inhibition studies.

b. Tryptic peptide analysis of Karimabad virus structural and non-structural proteins.

We have used two-dimensional peptide analysis to determine if viral proteins identified by immunoprecipitation contain shared amino acid sequences. To obtain sufficient quantities of Karimabad virus-coded proteins, two procedures were used, both of which rely upon immune selection. Since it was desired to examine nonstructural as well as structural proteins, infected cell lysates were used as a source of viral proteins. Six 150 cm^2 flasks of vero cells were infected and

labeled with 150 µCi/ml of ^{3}H-leucine. Lysates were then prepared, pooled, and divided into two aliquots. Viral proteins from one aliquot were selected by immuno-affinity chromotography using Affi-gel-10 (Bio-Rad) to which was coupled the immunoglobulins prepared from hyperimmune mouse ascitic fluids. The other aliquot was divided into four samples and the viral proteins collected by direct immunoprecipitation with varying antibody to antigen ratios to assure equivalence zone precipitation. These two alternative procedures gave essentially equivalent results.

Labeled viral proteins collected in this manner were solubilized in SDS-containing sample buffer and resolved on a 3 mm thick preparative polyacrylamide gel. Based on fluorographic exposures, the radioactive bands were excised from the gel, repeatedly washed with methanol to remove SDS and some of the scintillant, cut into small pieces and lyophilized. 50 µg/ml of TPCK-trypsin (Worthington) was added to the dried gel slices and incubated at 37°C for 24 hours. The peptides eluted from the gel slices were concentrated by lyophilization and resuspended in 50 µl of electrophoresis buffer (acetic acid: formic acid: H$_2$O-15:5:80). Aliquots of these samples from the Karimabad 74K, 31K, GP-58-60, GP-51, and nucleocapsid proteins were spotted on to cellulose thin layer chromatography plates (EM Reagents), electrophoresed in the first dimension, and chromatographed in the second dimension with a solvent buffer containing butanol: pyridine: acetic acid: H$_2$O (33:25:5:20) and 7% PPO (Fisher Chemicals). These plates were then dried and exposed to "pre-flashed" X OMAT R film (Kodak). The help of Dr. P. Allen (Frederick Cancer Research Center) in these studies is gratefully acknowledged.

Peptide maps were thus produced from all of the proteins listed above, except P-74 in which insufficient activity was obtained. The finger print from the GP-51 protein (see Figure 11) contained some, but not all of the peptides derived from the major virion glycoprotein band. As 1) the experiments with tunicamycin have shown that the major glycoprotein band consists of two distinct polypeptides, one of which has a similar mobility to the 51K polypeptide when synthesized in the presence of tunicamycin, and 2) the 3A8-7 (GP-2) monoclone reacts with both GP-58-60 and GP-51, the peptide data supports the view that GP-51 is an underglycosylated form of one of the Karimabad glycoproteins. No significant peptide homology was detected between these proteins and the 31K nonstructural protein or the nucleocapsid protein (data not shown).

However, a comparison of the peptide maps of the 31K protein and the nucleocapsid protein (24K) revealed two major, and at least one minor peptide that appeared to have similar migrations under the conditions used, and in fact migrated together in a mixing experiment. These maps are shown in Figure 7. Nonetheless, it is also apparent that most of the peptides from these two proteins are unique. Before discussing the possible interpretations of the apparent limited homology between these two proteins, it is useful to list the characteristics of the 31K polypeptide determined from previous experiments. The relevant data are as follows:

1. It is a nonstructural polypeptide.

2. A protein of similar molecular weight is also produced by several other sandfly viruses (Section IV-2).

3. In kinetic experiments, the synthesis of the 31K and nucleo-
    capsid proteins are initiated concurrently and at very early
    times after infection (2-3 hours) and 4-5 hours before the
    glycoproteins can be detected (preceeding annual report).

4. It is nonglycosylated and its migration is not affected by
    tunicamycin (Section IV-3).

5. It is labeled in a five minute pulse and is metabolically
    stable.

6. It is not detected if infected cells are labeled in the pre-
    sence of canavanine (previous annual report).

7. Its concentration is reduced relative to the nucleocapsid
    protein in the presence of zinc ions (see below).

8. It does not become membrane bound as determined from an
    analysis of membrane vesicles formed by homogenization of
    infected cells (preceeding annual report).

9. It is synthesized efficiently and rapidly in _in vitro_ transla-
    tion systems, based either on rabbit reticulocyte or wheat germ
    lysates (see below).

Although intriguing, the interpretation of the apparent homology
between these two proteins is not immediately apparent. At present, it is possible
that the co-migration of these peptides is simply a coincidence. Experiments which
have been conducted with the V-8 protease using the procedure of Cleveland (30)
have generated only 4 or 5 peptide bands which are poorly resolved. Therefore,
two-dimensional peptide mapping experiments are being repeated using chymotrypsin-
induced cleavage to confirm the results produced with the use of trypsin. It does,
however, seem clear that the 31K protein is _not_ a direct precursor to the nucleo-
capsid protein since the lower molecular weight nucleocapsid protein contains many
tryptic peptides not included in the larger molecular weight, 31K polypeptide.
Similarly, the 31K can _not_ be a "readthrough" product of the nucleocapsid protein,
or a phosphorylation variant.

If further experimentation confirms the existence of shared amino
acid sequences between these two proteins, then it logically follows that they are
coded by the same genome segment and at least partially in the same reading frame.
However, since the molecular weight of the shared peptides is unknown, the percen-
tage homology cannot be accurately determined from these data. Given these restric-
tions, and those noted above (i.e., that readthrough and direct precursor models
are eliminated), there are several theoretical models which could be offered to
account for the shared sequences.

1) The 31K and the nucleocapsid protein originate from a common precur-
sor polypeptide (polycistronic transcription) and a wobble in cleavage occurs during
its processing. Such abnormal processing has been found in other viral systems (31,
32,33). The only evidence to support this theory comes from studies in which it was
shown that the two polypeptides are detected concurrently and that in the presence
of all four amino acid analogues, both the nucleocapsid and 31K polypeptide are no
longer detected, whereas the GP-58-60 glycoproteins continue to be synthesized.
However, a precursor of the expected molecular weight was not detected - perhaps
because it migrated with GP-58-60 or perhaps because it was no longer immunopre-
cipitated. Such an irregular cleavage would also generate two additional peptides

in the area of the core band (see Figure 3 for Punta Toro virus and Figure 10 for Karimabad virus).

2. These proteins are coded by the same segment but mono-cistronic messengers are produced, one of which is internally initiated at a site on the negative strand within the sequence of the other. This model would require that in the synthesis of one mRNA, transcription proceeds through a stop signal recognized in the synthesis of the other message.

3. The above two models require that the nucleotide sequences coding for the putative shared amino acid sequences in the 31K and nucleocapsid protein be adjacent to both genes, that is they must lie between the unique sequences of the 31K and nucleocapsid protein. An alternative possibility exists in which the shared sequences are adjacent to only one (or neither) of the unique sequences and are juxtaposed by messenger splicing in the formation of mature messengers. The smallest genome segment of influenza virus is transcribed in this manner producing two mRNA's with some homologous and some heterologous sequences (23) Messenger splicing, however, is presumably a nuclear event, and although it is known that influenza transcription can occur in the nucleus, this has not been demonstrated for bunyaviruses. However, Pringle has reported that Bunyamwera virus does not replicate in enucleated cells, and fluorescent antibody studies have revealed apparent nuclear staining in both Punta Toro virus-infected cells (1979 Annual Report) and in the Rift Valley Fever virus-infected cells (34,35).

Although the simultaneous appearance of these two proteins in kinetic studies, and their simultaneous disappearance in the presence of a mixture of four amino acid analogues, as well as the peptide mapping experiments indicate that the synthesis of these two proteins is in some way related, it is unclear at present what this relationship could be. It was hoped that the production of monoclonal antibodies to Punta Toro virus would contain some clones which would precipitate both of these proteins. In fact, of 194 clones which react with Punta Toro virus nucleocapsid, only two react against the Karimabad nucleocapsid protein and neither of these appear to efficiently precipitate the 31K protein. However, the peptide data suggest that if homology between these two proteins exists, it will be a relatively small proportion of these proteins as most peptides are unique. Thus, these two monoclonal antibodies could easily be targeted at unique regions of the capsid protein and possibly the same determinant. Antigen specific but polyclonal antisera will be prepared to both of these proteins by immunization with gel-purified proteins and cross-immunoprecipitation will be performed to test this possibility (see renewal application). It will be shown that both the 31K and nucleocapsid proteins can be synthesized in large amounts in in vitro translation (IVT) systems - as are other polypeptides believed to be virus-specific based on immunoprecipitation analysis. This system will be dis-cussed below and in the current proposal. But it should be noted here that this system (combined with gradient separation of mRNA's) should enable the size of the corresponding messengers to be determined and hence eliminate some of the alternative schemes posed above. IVT systems also show efficient synthesis of two polypeptides with molecular weights smaller than the nucleocapsid protein which may be related to the processing of the 31K and nucleocapsid protein, as discussed above, and tryptic peptide analysis should help to clarify the inter-relationship of these IVT polypeptides.

c. Treatment of infected cells with zinc ions

Zinc ions are known to inhibit the activity of certain proteases and have been useful in studying the cleavage patterns of precursor proteins in several systems, notably picornaviruses (16), alphaviruses (17), and several normal cell proteins and glycoproteins. In the preceeding report we presented data which showed that, in the presence of 1.0 mM zinc chloride, Karimabad virus-infected cells accumulated a high molecular weight unglycosylated polypeptide with an approximate molecular weight of 105,000 daltons. This polypeptide was immunoprecipitated and not seen in similarly treated uninfected cells. However, this protein could not be "chased" (as has been noted in other zinc-inhibited precursors, 17), and in the absence of peptide analysis, its identity could not be determined.

Recent data, however, support the suggestion that this polypeptide may, in fact, be a precursor to the two Karimabad glycoproteins. We have carried out similar experiments with Punta Toro virus and have found that a similar polypeptide, but of slightly larger molecular weight (approximately 110,000 daltons), can be detected under the same conditions described above for Karimabad virus. On the basis of the data described in Section IV-3 in which the molecular weights of the unglycosylated envelope proteins of Karimabad and Punta Toro were determined in the presence of tunicamycin, a 5-7,000 dalton difference would be expected in a precursor produced by these two viruses, which in fact was found. The difference in the calculated versus the observed molecular weights (115,000 vs. 110,000 daltons for Punta Toro virus and 110,000 vs. 105,000 daltons for Karimabad virus) is likely due to the fact that on the 13%, DATD-cross-linked polyacrylamide gels used, proteins of this size are out of the linear region of molecular weight plots and thus their size is underestimated.

A second effect of zinc ions on the polypeptide profiles generated from immunoprecipitations of Karimabad virus infected cells has also been noted. Specifically, that the amounts of the 31K nonstructural polypeptide is reduced and that there is an increase in the labeling of a 47-48,000 polypeptide. Although it is tempting to speculate that these two events are related, and the increase in the 47K polypeptide is not seen in parallel experiments with uninfected cells, this peptide is uncomfortably close to the molecular weight of actin (43-46,000 daltons) which is known to adhere to immunoprecipitates.

Therefore, to eliminate the possibility that this 47-48K polypeptide is of host origin, the following mixing experiment will be performed. A lysate from zinc-treated and labeled uninfected cells will be prepared. This lysate will be mixed with a similarly prepared lysate from infected cells, but which has not been labeled. Immunoprecipitations will then be performed as usual, the results of which will be compared with those presented above.

If the 48K polypeptide is a precursor to the 31K nonstructural protein, there should also be a 17,000 molecular weight polypeptide fragment produced from this cleavage. Although such a polypeptide has not been found in Karimabad infected cells (and could be degraded), such a peptide is in fact immunoprecipitated from in vitro translations of Karimabad mRNA (next section). It should also be noted that the nucleocapsid proteins of the California group bunyaviruses is known to be coded by the small genome segment (36). This segment for Karimabad

virus has an apparent molecular weight of 0.8×10^6 daltons (4). If the small RNA segment for sandfly viruses also codes for the nucleocapsid protein, this segment would just accomodate the 48K polypeptide described above, and this relationship could explain the similar kinetics of synthesis of the 31K and nucleocapsid proteins.

d. In vitro translation of Karimabad virus RNA

As indicated in the previous proposal, in vitro translation (IVT) experiments with sandfly fever virus messenger RNA's would be initiated to study several aspects of the proteins specified by these viruses and the manner in which these were synthesized. Specifically, it was hoped that such studies would provide information with respect to:

1) whether polypeptides could be detected which were not identified in immunoprecipitates made from lysates of infected cells.

2) the size of the messenger RNA's coding for the recognized proteins. Separation of messengers could be achieved by density gradient or gel resolution followed by in vitro translation of appropriate fractions.

3) the identification of precursor polypeptides. As it is often the case that polypeptides are not cleaved in IVT systems (16,38), such precursors may be more easily recognized than in intact cells where cleavage may occur co-translationally. Alternatively, as some viral precursor polypeptides are cleaved (apparently due to the translation of viral specific proteases (24,37), such reactions could be studied more accurately where labeling times measured in seconds can be achieved.

4) the gene coding assignments of individual negative strand segments. Using the "translation arrest" procedures described by Inglis, et al., purified and separated negative strands are annealed to messenger RNA prior to translation, thereby inhibiting the translation of poly-peptides coded by this segment (see renewal proposal).

The data presented below will demonstrate that mRNA can be extracted from Karimabad virus-infected cells and translated in rabbit reticulocyte lysates with the production of large amounts of the 31K nonstructural protein, the nucleo-capsid protein, and moderate amounts of several other polypeptides which have not been detected in previous immunoprecipitations from vero or BHK cells. The virus specificity of these proteins has been demonstrated by a comparison of the poly-peptides translated from RNA extracted from infected and uninfected cells, and also by immunoprecipitation.

Total cytoplasmic RNA was extracted from five 150 cm^2 flasks, as described by Lai, et al. (39), with minor modifications. Cells were infected with a multiplicity of 5 and incubated for 18 hours. This time corresponds to periods of maximum viral protein synthesis, as determined from previous experiments. In

some cultures ^{3}H-uridine was included at 50 µCi/ml to facilitate the monitoring of RNA extraction. RNA extraction was performed at or below 4°C and all solutions and glassware were autoclaved to reduce the effects of ribonucleases. The cell cultures were washed three times in ice-cold TNE buffer (0.1 M NaCl, 0.01 M Tris, pH 7.4, and 0.001 M EDTA) and drained. 1.5 mls of 0.5% NP-40 detergent (Bethesda Research Laboratories) in TNE buffer was added per flask and incubated for 20 minutes. The resulting lysates were pooled, gently homogenized, and nuclei were removed by centrifugation at 2500 x g for 10 minutes. The supernatant was made 0.5% with respect to SDS, and an equal volume of buffer-saturated phenol-cresol (containing 8-hydroxy-quinolone) was added and the phases separated by centrifugation.

A second phenol extraction followed by chloroform extraction resulted in a final aqueous phase from which RNA was precipitated with 2 volumes of ethanol and 0.06 volumes of 5M LiCl. The extracted RNA was solublized in LiCl containing buffer, reprecipitated with ethanol, dissolved in H_2O and aliquots stored at -80°C. LiCl extractions were performed to remove double-stranded RNA which is known to inhibit IVT systems. A vanadyl ribonucleoside RNase inhibitor (Bethesda Research Laboratories) was used in some extractions but did not appear to alter the results obtained. RNA yields and concentrations used in IVT experiments were estimated by optical density determinations. In the experiments described below, this RNA was used without further fractionation.

As the suppression of host-cell specific protein synthesis is both delayed and incomplete in cells infected with Karimabad virus, it was recognized that extracted cellular messengers would also be active, however, the virus specificity of translated polypeptides has been confirmed by immunoprecipitation. Actinomycin D, added two hours after infection, was used in attempts to reduce the background level of cellular polypeptides, but was found to be generally ineffective - despite a 90% reduction of ^{3}H-uridine incorporation into extracted RNA. Presumably, the cellular polypeptides were translated from long-lived mRNA's or alternatively actinomycin D is less efficient in inhibiting messenger RNA transcription than that of other classes.

The rabbit reticulocyte system has been chosen for most of our studies as it has been shown to efficiently translate high molecular weight messengers from a variety of different sources and has a low endogenous activity if pretreated with micrococcal nuclease to inactivate endogenous mRNA's. The effect of altering the concentrations of K^+, Mg^{++}, and RNA concentrations was assessed, but found to give essentially equivalent results over broad ranges. Our standard assay is done at 30°C in 3.5 mM Mg^{++}, 115 mMK$^+$, and 25 µg of RNA from infected or uninfected cells. The total volume of each assay is 30 µl which includes 20 µCi of 35S-methionine. The wheat germ lysate has also been compared to the rabbit reticulocyte lysate but found to be much less efficient in translation and has not generated any polypeptides which were not also seen in the rabbit reticulocyte system. Both translation systems were purchased from Bethesda Research Laboratories.

Several RNA extractions have been performed and these preparations have, for the most part, been shown to direct the synthesis of similar polypeptides. However, some differences have been noted and the results generated from two such preparations will be shown. Figure 8 shows the kinetics analysis of polypeptide synthesis with 20 µCi of 35S-methionine per 30 µl translation assay, and with RNA

extracted from infected or uninfected cells. At the times inidcated, 3 µl of each
lysate was removed, mixed with SDS-containing electrophoresis sample buffer, and
analyzed directly on gels.

With this level of ^{35}S-methionine, viral polypeptides are first
detected at approximately 10 minutes and continue to be synthesized for several
hours. As can be seen from this figure, there are 5 unique polypeptides which
are synthesized in response to the RNA extracted from infected cells. They are
the 31K nonstructural protein, the 24K nucleocapsid protein, a 22K and a 17K poly-
peptide, and a polypeptide of molecular weight in excess of 100K, but whose
molecular weight cannot be accurately determined from gels of this concentration
(see Figure 10 and 11 for comparison to the migration of known Karimabad viral
proteins).

Immunoprecipitation analysis of these polypeptides with various
dilution of Karimabad HMAF demonstrates that each of these polypeptides whose
synthesis is stimulated only from infected cell RNA are reactive with this anti-
sera (Figure 9). The high molecular weight protein is not efficiently synthesized
until 90 minutes after the addition of the RNA, and its existence was therefore
not noted in earlier experiments. The 22K protein is not believed to be an arti-
fact of in vitro translation (premature termination product) as a polypeptide of
identical molecular weight is detected in immunoprecipitates from Punta Toro
infected cells (Figure 3) and occasionally in cells infected with Karimabad.
The 17K polypeptide will be discussed below.

Polypeptides with migrations equivalent to the mature virion
glycoproteins, and reactive to specific antiserum, have not been detected. The
high molecular weight polypeptide could be an uncleaved precursor to these pro-
teins, but its molecular weight appears to be higher than the polypeptide which
accumulates in zinc-inhibited cells. Conceivably, it is an earlier precursor or
perhaps represents the virion-associated transcriptase, which is necessary due
to the negative-stranded nature of these viruses. Reaction with monoclonal
antibodies and tryptic peptide analysis will be required to resolve these ques-
tions, and are discussed in the accompanying proposal.

Figure 10 shows the immunoprecipitated polypeptides synthesized
in response to different preparations of RNA which had been extracted from
infected or uninfected cells, treated or untreated with actinomycin (2 µg/ml).
Varying amounts of RNA were added as indicated in the figure and incubated for
4 hours. The 17K, 22K, 24K and 31K polypeptides were detected as shown above
and again are not present from translations primed with uninfected cell RNA.
Comigration of the 31K and 24K polypeptides with the equivalent proteins immuno-
precipitated from intact vero cells is shown. The high molecular weight poly-
peptide has not been detected in translations with this RNA preparation, however
two additional polypeptides of 48K and 65K are seen which are not apparent in the
uninfected cell RNA translations. The 48K comigrates with the protein discussed
above in zinc-inhibition studies. Again, the significance of this finding is
complicated by the binding of actin to immunoprecipitates. Therefore, the
experiments proposed above with respect to the zinc-inhibited protein must also
be carried out in these experiments. It should, however, be noted that the 17K
polypeptide and the 31K polypeptide may well be formed from the cleavage of the
48K polypeptide, and that these polypeptides, plus the core polypeptide, could
constitute the total coding capacity of the S genome segment. Alternatively, the
48K polypeptide could be composed of the 22K and 24K (nucleocapsid) with the
remainder of the small genome segment coding for the 31K polypeptide.

In Figure 11 is presented the results from pulse-stop experiments carried out in the presence of 4 times the normal level of ^{35}S-methionine, and for the times indicated (30", 1', 2', 10'). As can be seen from this figure, the 31K, 24K, and 22K are detected even in these very short labeling periods. This would indicate that these polypeptides are translated from individual messengers, or alternatively that cleavage is, indeed, a cotranslational event. As discussed in the accompanying application, chymotryptic and tryptic peptide analysis will be carried out on these IVT polypeptides and their reactivities to monoclonal and polyclonal antisera will be determined to assess their interrelationships. Microsomal membranes will be added to these translation lysates to determine if, under these conditions, the envelope proteins are efficiently synthesized or per-haps cleaved from a precursor. Also proposed are gradient fractionation (size estimation) of the mRNA's which code for these polypeptides and translation arrest experiments to determine gene coding assignments.

If the high molecular weight IVT protein can be shown to contain tryptic peptides or antigenic determinants in common with the virion glycoproteins this would 1) indicate that these proteins are translated as a precursor, 2) support the tentative conclusions based on zinc-inhibition data, and 3) explain why such proteins have not been detected directly in our translation lysates. If this protein is distinct from the virion glycoprotein, then modifications will have to be made in either our RNA extraction procedures or our translation conditions to effect translation of these species,since it is known that the glycoproteins were being synthesized in the intact cells at the time of RNA extraction. It is ex-pected that the sum of these data, collected from both in vitro translation and from studies of intact cells will enable us to describe the mechanisms by which sandfly fever viruses control the expression of their genomes.

6) Initiation of Animal Studies to Evaluate In vivo Functions of Specific Viral Proteins and Antigenic Determinants

a. Foreword

In the previous application, it was proposed that animal studies be initiated in the last half of the current contract year for the specific purposes described below. Due to 1) the availability of specific monoclonal antibodies earlier than was expected, and 2) the availability of a graduate student (D. Pifat) who desired to work on these problems, these studies have been initiated earlier than was proposed, and consequently a more complete introduction (borrowed from the accompanying application) is provided. These experiments are not intended to constitute a major shift away from our biochemical characterization of these viruses. Rather,they are seen as an extension of these studies, often requiring similar methodology, in assessing the biological functions of sandfly virus pro-teins, especially as they are relevant to immunoprophylaxis.

b. Introduction

Animal infection experiments have been carried out in an attempt to establish a laboratory model for the infections produced by at least some of the classical sandfly fevers. Ideally, such infections could be established in adult (fully immunocompetent) inbred and outbred animals and be produced by peripheral challenge. For reasons which will be summarized below, mice have been selected as a laboratory model and Punta Toro virus as a representative sandfly agent.

We do not intend to imply that the induction of a sandfly fever virus infection in mice is fully equivalent to the disease as seen in humans. Neither is it our intention to pursue extensive basic studies in animal pathogenesis nor basic studies in the various immunological effector mechanisms which may contribute to the resolution of the acute disease process. However, we do believe that a mouse model is adequate to address at least some of the questions which relate to the immunogenicity and protective capacity of the viral proteins which we have characterized in past and ongoing in vitro studies. It is hoped that such studies would contribute to the rational design and construction of vaccines intended for human immunoprophylaxis.

As indicated in the previous proposal, the major objectives of these studies are 1) to define for this group of viruses the biological role of specific viral proteins in providing immunologic protection in vivo, and 2) how such protective responses can be most efficiently stimulated. This can, and will be addressed, both by the active immunization of animals with purified proteins and by passive immunization with (monoclonal and polyclonal) antibody preparations of predetermined specificity. It was also proposed to produce inactivated or glycoprotein vaccines, as described by Morein, et al., who have shown that such preparations are effective in providing protection in very minute doses, provided that they are presented to the immune system as micelles rather than as monomeric proteins (40).

The availability of monoclonal (MC) antibodies which we have characterized in the current contract year (in collaboration with Dr. Joel Dalrymple, USAMRIID) is expected to greatly facilitate the objectives summarized above.

First, MC-antibodies can be administered directly to animals to determine their ability to alter the outcome of ongoing or subsequent infections, and by immunoprecipitation and gel analysis (described in Section IV-4), the target antigenic determinants can be traced to specific viral proteins. Apriori, the administration of such preformed immunoglobulins could result in protection, enhancement, or show no effect. Since MC-antibodies are, by definition directed at a single determinant, the demonstration that a passively transferred MC-antibody is capable of altering the outcome of a disease process is highly significant in terms of the theoretical effect expected from that determinant in active immunization. Such in vivo biologically active monoclones are also of practical use in the evaluation of experimental immunogens. Potential vaccines of any type (attenuated, inactivated, or subunit) including those produced by recombinant DNA technology (as has been suggested for Rift Valley Fever) can be screened for their capacity to react with protecting MC-antibodies or capacity to induce antibodies capable of competing with such MC-antibodies in competitive binding assays.

Second, the high titer and specific reactivities of these immunoglobulins can be utilized to purify relatively large quantities of undenatured viral proteins (by immuno-affinity techniques), which are demonstrably free of other viral or host antigens. The efficiency of this technique has been documented in the 1979 Annual Report in which immunoglobulins from hyperimmune ascitic fluids were covalently coupled to affinity supports. Immuno-affinity purification is of particular value in studies with sandfly fever viruses which replicate to relatively low titers in vitro and which tend to be unstable in gradient purification techniques.

Monoclonal antibodies are also very useful in determining the correlation between specific viral proteins and in vitro functions such as plaque-reduction neutralization and hemagglutination-inhibition. However, there is growing evidence that protection experiments derived from data collected in vitro (neutralization) does not always accurately predict functional protection in vivo. For example, MC-antibody directed at Sindbis virus E_2 with demonstrable neutralizing activity was shown to be less able to protect mice than another monoclone directed at E_1 with no neutralizing activity (Cole, Johnson, Dalrymple, personal communication). Also, the sandfly virus, Arumowot, which does not cross-react with Rift Valley Fever virus in in vitro neutralization tests, is capable of providing some actively-induced immunity against Rift Valley Fever virus challenge, whereas Punta Toro virus which does show heterologous neutralization activity with Rift Valley Fever virus provides no protection (42). Finally, data presented below will show that a Punta Toro virus-specific MC-antibody protects mice from a lethal infection by Punta Toro virus, while PTV hyperimmune ascitic fluid with twice the neutralization index of the monoclone is less efficient in this respect. It is also recognized that the generation of "incomplete immunity" by some non-replicating vaccines (e.g., measles and respiratory syncitial virus), despite the neutralizing activities induced, may lead to greatly increased pathology, mediated by hypersensitivity reactions, when natural challenge occurs. In summary, these studies indicate that protection in vivo does not necessarily correlate directly with in vitro neutralization activity, and documents the need for an animal model in assessing the relevant antigens in, and the immune response desired from, experimental immunogens.

c. Selection of Animal and Virus Strains

Mice have been selected to initiate animal infection experiments in consideration of 1) expense, 2) ease of handling, 3) availability of defined inbred strains, 4) the broad existing knowledge of the murine immune system, 5) commercial availability of murine-specific immunologic reagents, 6) the early results of Sabin which demonstrated that Sicilian and Naples sandfly viruses could be adapted to mice (42), and 7) the fact that the virus-specific monoclones are of murine origin.

Punta Toro virus initially has been chosen over other sandfly agents as 1) it is a recognized human pathogen, 2) we have focused on this virus (as well as Karimabad virus) in our in vitro studies in which the characterization of viral proteins and glycoproteins has been a major emphasis, 3) a panel of MC-antibodies is available, some of which react with viral envelope antigens, 4) the two viral envelope glycoproteins of PTV readily separate by our standard electorphoretic procedures thus allowing independent and relative analysis of their biological role in infection and ability to induce immunologic protection, and 5) several different strains have been isolated, which although they generate nearly identical polypeptide profiles on gel analysis (Figure 3), do differ significantly in their virulence for hamsters.

d. Results and Discussion (Tables are presented at the end of this section)

1. Susceptibility of mice to Punta Toro and Karimabad sandfly viruses.

The prototype strain of Punta Toro virus (PTV-P), which had been adapted to vero cells, was obtained originally from the Walter Reed Army Institute of Research and was passaged 3 times in vero cells prior to its inoculation into 2-4 day old outbred ICR mice. These mice were inoculated intracerebrally (i.c.)

with 0.02 ml of a 10^{-1} dilution of vero adapted virus containing approximately 2×10^6 pfu/ml. Suckling mice were found to be uniformly susceptible to this virus. Brain tissues from moribund animals were pooled and homogenized in Eagle's medium containing 100 units per ml of penicillin and 100 μg per ml of streptomycin. Tissue homogenates (10%) were clarified at 10,000 x g for 20 minutes and stored at -80°C. A second i.c. passage in suckling mice yielded a homogenate (designated SMB-2) with approximately 1×10^7 pfu/ml, corresponding to approximately 10^8 pfu/gram of brain. However, in titration of this material in suckling mice, 0.02 mls of a 10^{-6} dilution killed all inoculated animals, indicating that the true infectious titer is higher than that indicated by titration in vero cells.

Based on the results reported by Sabin with Naples and Sicilian strains of sandfly fever (42), in which 35 and 10 serial passages in suckling mice were required, respectively, for the isolation of strains lethal to adult mice, it was expected that similar adaption could be required for Punta Toro virus (PTV). This, however, was found not to be the case (Table 6). Both the SMB-2 and the original tissue culture adapted virus induced uniformly lethal infections when inoculated intracerebrally into adult ICR mice, although mean survival times appeared less in animals inoculated with the brain adapted virus. Moribund mice exhibited hind-leg paralysis, curvature of the thoracic spine, and muscle clonus indicative of CNS disease. Ocular inflammations of varying severity and loss of equilibrium are also commonly observed in dying animals. Fluorescent antibody staining of cryostat sections with hyperimmune ascitic fluid showed multiple foci of fluorescence throughout the cerebral cortex with an accumulation of antigen in the pyramidal cells of the hippocampus, thus demonstrating an active encephalitis with Punta Toro virus.

In contrast to the reproducibly fatal infections following i.c. inoculations, intraperitoneal (i.p.) infections with tissue culture or brain adapted PTV were unable to kill mice at any dilution unless infection was initiated during the first week of life (Table 6). An unknown, but dramatic change occurs during the second week which renders mice resistant to i.p. challenge. Such an abrupt alteration in susceptibility following peripheral challenge is not unique to this system, but has been noted also with togaviruses, rhabdoviruses, herpesviruses, coxackiviruses, and coronaviruses, and has been attributed, at least in part, to maturation of macrophages (43). Very similar results have been obtained in experiments with Karimabad virus (adapted to vero cells) in which mice of any age were shown susceptible to i.c. inoculation but only young animals are killed by peripheral inoculation (Table 7).

Two inbred mouse strains were also examined for their relative susceptibility to i.c. and i.p. inoculations with the prototype strain of Punta Toro virus (SMB), and were found not to differ significantly (Table 8). Adult C57BL/6J and CBA/J strains were resistant to i.p. inoculation at any dilution of virus. Inoculation of a 10^{-2} dilution of virus, i.c., was lethal for all mice, whereas a 10^{-4} dilution resulted in extended survival times and some survivors.

Intraperitoneal inoculation does, however, provide at least partial protection against an homologous i.c. challenge. In several experiments adult mice of several strains were given 0.1 ml of a 10^{-1} dilution of PTV-P (SMB), and approximately 50% of these animals were found to be resistant if challenged i.c. at two to three weeks. Similar experiments with vero-adapted Karimabad virus resulted in greater than 90% survival following i.c. challenge. Based on this data, it was

considered likely that mice may be susceptible to an i.p. challenge if they were immunosuppressed, and that unrestricted replication of peripherally inoculated virus could result in adaption and selection of a variant which would be lethal following peripheral inoculation in the absence of immunosuppression. However, using cyclophosphamide (cytoxan) as an immunosuppressive agent (200 mg/kg), this was not observed. Of 20 adult ICR mice given cytoxan at 24 hours or 24 and 48 hours after i.p. infection with Punta Toro virus, none developed any observable illness. These mice, however, were susceptible to i.c. challenge at day 20, indicating that the usual protective immune response induced by i.p. inoculation had, in fact, been prevented by cyclosphosphamide treatment. Apparently, mice are able to contain the replication of Punta Toro virus inoculated intraperitoneally by mechanisms unrelated to those which establish protective immunity.

In summary, in an attempt to develop an in vivo system to pursue the objectives described above, our experience with several strains of mice has indicated that adult animals are reproducibly susceptible to an infection initiated directly in the brain, but that an undefined alteration occurs early in life which renders them resistant to peripheral inoculation. As a model for human disease this system is clearly limited, as natural infections of humans with Punta Toro virus are initiated peripherally by the sandfly vector and are not known to produce encephalitis. However, as a model in which to evaluate the role of individual viral proteins in providing immunologic protection, i.c. challenge in mice represents a rigorous test, and has shown considerable promise as described below.

2. Effect of passive transfer of monoclonal antibodies on PTV infections in mice.

As indicated elswhere in this report, hybridomas which produce antibodies directed at PTV antigens have been isolated and the individual target proteins have been determined by immunoprecipitation analysis. Five of these monoclonal antibodies react specifically with determinants on viral envelope glycoproteins, 4 react against GP-2 and one against GP-1. The remainder of the clones react with determinants present only on the internal capsid protein. The effectiveness of the 5 monoclonal antibodies which recognize glycoprotein determinants in preventing the fatal encephalitis in mice described in the preceeding section has been tested and compared with polyclonal hyperimmune mouse ascitic fluid and a monoclone specific for the nucleocapsid protein. The results are presented in Table 9.

Mice were passively immunized with 0.1 ml of undiluted individual ascitic fluids 4 hours before and 24 hours after i.c. inoculation of approximately 100 LD$_{50}$ units of Punta Toro virus (SMB-2) or the heterologous sandfly virus, Karimabad. The mice were then monitored for 1 month to determine the degree of protection based on survival and mean day of death. As shown in Table 9, none of the GP-2 specific monoclones or the nucleocapsid-specific monoclone affored any protection as compared to control mice. In direct contrast, the GP-1-specific monoclone (7F5-7) protected all mice which exhibited no deaths and no illness.

Although 7F5-7 (GP-1) does possess the highest neutralizing titer among the available monoclones, it cannot be concluded from these experiments that neutralization activity, per se, as determined in vitro, is sufficient to provide protection, or even that protection correlates directly with neutralizing activity. This follows from the observations that 9G6-7 (GP-2) which neutralizes

PTV-P in vitro does not delay death relative to controls, and that the hyper-immune ascitic fluid, which has twice the neutralizing capacity of 7F5-7 (GP-1), affords only a delay in mean survival time, with all mice eventually progressing to death.

It is not yet known whether 7F5-7 (GP-1) if given after virus infection will protect, i.e., if all virus must be neutralized upon injection or whether this antibody, given passively, will resolve a disease process pre-viously initiated. However, we do know that the animals protected with 7F5-7 (GP-1) did not make antibody to the PTV GP-2 protein. At 2, 3 and 4 weeks after virus infection, 30 μl of blood was removed from each animal, pooled, and reacted with a ^{3}H-leucine-labeled lysate of PTV-infected vero cells in our standard immuno-precipitation assay. The resulting gel profiles showed a stong nucleocapsid band and GP-1 in all time samples. The anti-nucleocapsid activity clearly must have been endogenously produced, although the GP-1 activity could have been a result of antigen stimulation or residual 7F5-7 (GP-1). However, as we know that this monoclone is IgG (reacts with Protein A), that the average half-life of mouse IgG is isotype dependent but averages 4 days (44), and that effective immunoprecipitation with this hybridoma can be accomplished with a dilution of 1:600, it does not seem necessary to invoke endogenous GP-1 antibody synthesis to account for this activity. Therefore, although other interpretations are possible, the most straightforward interpretation of these results is that anti-GP-2 activity was not induced due to a failure of the virus to replicate and conse-quently that the 7F5-7 (GP-1) monoclone successfully neutralized the virus inocu-lum or initial multiplication cycles. Endogenous antibody production to the nu-cleocapsid protein was likely due to immunogenic quantities in the mouse brain inoculum, and to the apparent fact that it is the immuno-dominant antigen based on the high frequency of isolation of hybridomas to this antigen (93%).

None of these monoclones protected mice from the heterologous Karimabad virus despite the fact that 3A8-7 (GP-2) reacts with a Karimabad glycoprotein by immunoprecipitation. In fact, although the number of mice which could be studied was limited, due to the quantities of ascitic fluids available, mice inoculated with this cross-reacting monoclone died 3-4 days earlier than controls. These experiments are currently being repeated, but this result raises the disturbing possibility that some pre-existing antibodies could enhance the infection of hetero-logous sandfly viruses.

An interesting observation which is currently unexplained is that PTV-P HMAF which did not protect in the homologous challenge, when given in these amounts, did protect three out of five mice inoculated with Karimabad virus. Although PTV-P HMAF does immunoprecipitate at least one of the Karimabad glycoproteins (which do not adequately separate on our polyacrylamide gels), the Karimabad virus determi-nants recognized by PTV-P HMAF relative to the 3A8-7 (GP-2) monoclone are unknown. This result, however, is consistent with our observation that i.p. inoculation with infectious PTV-P results in partial protection of mice from an i.c. challenge 1 month later with Karimabad virus. As antiserum to PTV-P has not been shown to react with Karimabad virus in plaque reduction tests (46), these results again suggest, although based on limited numbers of animals, that results from in vitro protection and in vivo protection studies are not necessarily monitoring the same parameters.

The indications that arise from these studies are that pre-existing antibody reactive with viral surface antigens is sufficient to provide protection from a rigorous PTV-P in vivo challenge provided that it is directed at the relevant antigenic determinant, and that antibody to a single unique determinant is sufficient. Whether other determinants on GP-1 or GP-2 will be shown to provide protection in mice can be, but as yet has not been, determined. Nor is it known whether 7F5-7 (GP-1) could provide protection if administered after infection. Although the biological effect of preexisting antibodies to GP-2 cannot be assessed from negative data, and they may not be irrelevant in terms of protection, they do not appear to be a pre-requisite. However, in similar studies carried out in hamsters, Dr. C. J. Peters (USAMRIID) has shown that, in addition to 7F5-7 (GP-1), 9G6-7 (GP-2) also provides some protection, although it is less efficient (9). The factors which account for this difference are not definitely known since equivalent doses were administered, but may be related to the different route of virus infection used (i.p. vs. i.c.).

Data presented in Section IV-4 in this report suggest (although indirectly) that each of the GP-2 specific monoclones detects a separate determinant. However, we do not know how many determinants exist on these proteins for which we have no corresponding monoclones, and which may be of direct relevance to protection and immunoprophylaxis (or conceivably enhancement). Therefore, as indicated in the accompanying renewal application, other glycoprotein-specific hybridomas will be isolated, their specificities determined, and tested for their in vivo biologic effect. As a complement to these studies, GP-1 and GP-2 will be purified by immuno-affinity chromatography using existing monoclones, and antigen-specific but polyclonal hyperimmune ascitic fluid prepared. Biological activities of these antisera which are not represented in the battery of monoclones will indicate that relevant determinants are present which are not yet recognized by existing monoclones. It is also recognized that isotypic differences which may not be apparent by immunoprecipitation may have a profound influence on biological activities (e.g., complement and macrophage binding). Therefore, the monoclones will be identified as to isotype utilizing specific antisera in fluorescent antibody or immunodiffusion assays (45). The utilization of this in vivo system in the evaluation of various experimental immunogens and their reactivity with 7F5-7(GP-1) is also described in the renewal application. If the GP-1 determinant which is recognized by 7F5-7 is shown by further experimentation to be the major determinant capable of providing protection, the isolation of this clone was very fortuitous.

e. Notes Added in Proof:

1) In recent experiments, we have reproduced the results which demonstrate the protecting capability of 7F5-7 (GP-1), and have also demonstrated that homologous hyperimmune ascitic fluid, passively transferred, is also protective if given in larger amounts. However, the conclusion remains that HMAF is much less effective than 7F5-7 (GP-1), despite its higher neutralizing titer. In addition, we have shown that 7F5-7 (GP-1) is capable of providing complete protection even if diluted 1:2 and administered after virus infection is initiated. These data are summarized below. The animals used were ICR adults (Flow Laboratories) which were inoculated i.c. with 100 LD_{50} units of vero-adapted PTV-P.

		Passive Transfer			
	Ascitic Fluid	Amount	Dilution	Time (Hrs.) (relative to virus inoculation)	D/T
1)	None	-	-	-	10/10
2)	7F5-7 (GP1)	0.2 ml	1:2	-4	0/10
3)	7F5-7 (GP-1)	0.1 ml	1:2	+24, +72	0/10
4)	HMAF (PTV-P)	0.2 ml	und.	-4, +24, +72	0/10
5)	HMAF (RVF)	"	"	"	10/10
6)	HMAF (Karimabad)	"	"	"	10/10
7)	HMAF (Candiru)	"	"	"	10/10

2) Also, in recent experiments we have found that one of the Punta Toro strains described above in Section IV-2 is capable of causing lethal infections in C57/BL/J adult mice following either intraperitoneal or subcutaneous inoculations. This system is expected to have several advantages over that described above, and is discussed in the accompanying application.

Table 6

Infection of ICR Mice of Different
Ages with Punta Toro Virus (PTV-P)

Intracerebral Inoculation

| Virus | Age of Mice (Days) | Virus Dilution | D/T | Day of Death | |
				Mean	Range
TC-Vero[1]	2	10^{-1}	8/8	4.2	4-5
	6	10^{-1}	10/10	4.5	4-5
	Adults	10^{-1}	7/7	10.2	9-11
SMB[2]	2	10^{-4}	10/10	4.5	4-5
	2	10^{-6}	15/15	5	5
	10-12	10^{-2}	5/5	4.5	4-5
	Adults	10^{-2}	12/12	7.1	6-8

Intraperitoneal Inoculation

| Virus | Age of Mice (Days) | Virus Dilution | D/T | Day of Death | |
				Mean	Range
TC-Vero	2	10^{-1}	12/14	10.1	8-13
	5-6	10^{-1}	10/10	14.2	14-15
	10-12	10^{-1}	0/9	-	-
	Adults	10^{-1}	0/8	-	-
	2	10^{-2}	6/13	12	8-14
	2	10^{-5}	0/10	-	-
	10-12	10^{-2}	0/10	-	-
	40	10^{-2}	0/5	-	-
	Adults	10^{-2}	0/5	-	-

[1] Virus adapted to Vero cells. Titer=approximately 8×10^6 pfu/ml.

[2] Virus dilutions prepared from a 10% homogenate of infected suckling mouse brain containing approximately 1×10^7 pfu/ml.

Table 7

Infection of Mice With Karimabad Virus[1]

Intracerebral Inoculation

Mouse Strain	Age of Mice	D/T	Day of Death Mean	Range
ICR	10-12 days	6/6	4.3	4-5
BALB/c	Adults	4/5	6.7	6-8
C57/BL/J	Adults	6/6	7.8	6-9
CBA/J x C57/BL/J	Adults	6/6	8.9	7-11

Intraperitoneal Inoculation

Mouse Strain	Age of Mice	D/T	Mean	Range
ICR	10-12 days	12/12	5.9	4-7
ICR	4 weeks	0/8	-	-
ICR	Adults	0/6	-	-
C57/BL/J	Adults	0/8	-	-
CBA/J x C57/BL/J	Adults	0/6	-	-

Intravenous Inoculation

Mouse Strain	Age of Mice	D/T	Mean	Range
ICR	Adults	0/8	-	-

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[1] Virus adapted to Vero cells. Titer=approximately 2×10^7 pfu per/ml, used undiluted.

Table 8

Infection of C57BL/6J and CBA/J Inbred Mice
with Punta Toro Virus (PTV-P)[1]

Strain	Route of Infection	Virus Dilution	D/T	Day of Death	
				Mean	Range
C57BL/6J	i.c.	10^{-2}	6/6	11.5	7-16
		10^{-4}	3/5	13.6	12-15
		10^{-6}	0/4	-	-
	i.p.	10^{-1}	0/6	-	-
		10^{-3}	0/6	-	-
		10^{-5}	0/6	-	-
CBA/J	i.c.	10^{-2}	4/4	9	7-11
		10^{-4}	2/3	12	11-13
		10^{-6}	0/3	-	-
	i.p.	10^{-1}	0/6	-	-
		10^{-3}	0/6	-	-
		10^{-5}	0/6	-	-

[1]Virus dilutions prepared from a suckling mouse brain pool containing approximately 1×10^7 pfu/ml. Volumes inoculated: i.p. = 0.1 ml, i.c. = 0.03 ml.

Table 9

Effect of Passive Transfer of PTV-HMAF or PTV-Hybridoma
Ascitic Fluids on PTV-P or KV Inoculation (Intracerebral) of Mice[4]

Ascitic Fluid	Protein Specificity		Homologous Titer		Virus Inoculated[2]	D/T	Day of Death	
	PTV	KV	Neut[1]	HI[1]			Mean	Range
None	-	-	-	-	PTV	4/4	9	7-11
					KV	5/6	9	7-11
HMAF	GP-1, GP-2,	C,GP	13,600	320	PTV	5/5	15.6	12-20
					KV	2/5	10	9-11
1A10-1	C	ND[3]	<2	<10	PTV	5/5	9.4	8-11
					KV	5/5	10	9-11
4G2-6	GP-2	ND	2	160	PTV	6/6	13.6	8-18
					KV	5/6	9	8-10
4A9-6	GP-2	ND	24	320	PTV	5/5	8.8	8-9
					KV	5/5	8.8	8-9
9G6-7	GP-2	ND	2,000	1,280	PTV	5/5	9.2	9-10
					KV	4/5	8.7	6-10
3A8-7	GP-2	GP	2	640	PTV	5/5	8.4	5-10
					KV	3/4	5.6	5-6
7F5-7	GP-1	ND	6,300	320	PTV	0/5	-	-
					KV	4/5	7.7	5-10

[1]
80% plaque reduction neutralization titer or reciprocal of serum dilution inhibiting
8 HA units, determined at USAMRIID by Drs. Dalrymple and Peters.

[2]
Mice were inoculated intracerebrally with 0.03 ml containing approximately 100 LD_{50}
units of virus.

[3]
ND=none detectable by immunoprecipitation analysis.

[4]
Mice were CBA/J x F_1 (CBA/J by C57BL/6J), shown to be uniformly susceptible to i.c.
inoculation of both KV or PTV-P.

V. Studies to be Completed in the Current Contract Year

In the discussion of the data presented in this report, frequent reference
has been made to the directions which will be pursued in confirming and expanding
the observations made. The continuation of most of these experiments realistically
will require extension into the forthcoming year, and is so indicated in the
accompanying application. With the exception of the initiation of animal studies
(which have been proposed in previous applications), emphasis in the renewal
application has been placed on resolving the issues raised in this report rather
than to initiate new projects. Several studies, however, have been completed or
can realistically be expected to be completed in the current contract year.

1) The electron microscopy studies relating to the morphology and morpho-
genesis of sandfly fever viruses, much of which was reported in the previous
annual report, are now complete with the delineation of virion fine structure
which was made possible by virtue of improved gradient isolation techniques. These
studies have enabled us to define, in morphological terms, the interactions of
viral components which occur during virion assembly more precisely than previously
reported for these or other bunyaviruses. These studies also establish the basis
from which to examine these interactions by biochemical techniques.

2) The tunicamycin inhibition studies reported above have completed our
analysis of the glycosylation and glycopeptide structure of sandfly fever virus
envelope proteins, with the exception of oligosaccharide structures present on the
purified virion. As reported previously, the unusual absence of complex oligo-
saccharide units on virus glycoproteins extracted from infected cells is unique
among enveloped-viruses. Therefore, we feel that a similar analysis should be
conducted on purified and released virions to eliminate the possibility that our
selection of virus glycoproteins by immunoprecipitation from infected cells selects
for incompletely processed glycoproteins. Difficulties in this analysis are no
longer expected due to the apparent stability of sandfly viruses in renografin
gradients.

3) Our initial observations that the 7F5-7 (GP-1) monoclonal antibody does
not react with unglycosylated GP-1 produced in the presence of tunicamycin requires
confirmation due to the implications raised by this result and the fact that this
monoclonal antibody has high neutralization titers and is highly efficient in providing
protection to animals infected with Punta Toro virus. These experiments will
therefore be repeated and compared to the reactivity of this monoclone with de-
glycosylated GP-1 produced by endoglycosidase H treatment.

4) In our screening of several additional sandfly agents it was noted that
Candiru virus specified an 85,000 dalton polypeptide which appears to be a precursor
polypeptide as it is preferentially labeled in short labeling times and is meta-
bolically unstable. A protein of identical molecular weight has been detected in
Punta Toro virus-infected cells which were inhibited with amino acid analogues.
Why Candiru virus should be unique among the viruses so far examined is unknown.
However, if this polypeptide represents a precursor, which for some reason is
processed more slowly than in other sandfly virus-infected cells, this observation
would be experimentally useful in reference to our studies of transcriptional
controls and post-transcriptional processing. These studies will be approached
by pulse-chase experiments, tryptic peptide mapping. and reactions with Punta Toro
virus monoclonal antibodies, many of which cross-react with Candiru virus polypeptides.

5) As discussed in Section IV-6, there are several reasons which dictate the selection of Punta Toro virus in our _in vivo_ studies, and consequently we do rot intend to continue similar experiments with Karimabad virus. However, one observation should be pursued, specifically that animals infected with Karimabad virus, and given the 3A8-7 (GP-2) monoclone, died much earlier than control animals. If immunologic enhancement exists for this group of viruses, this information should be taken into consideration in the design of experimental immunogens. Therefore, this experiment will be repeated with sufficient numbers of animals to enable statistical evaluation of the results obtained.

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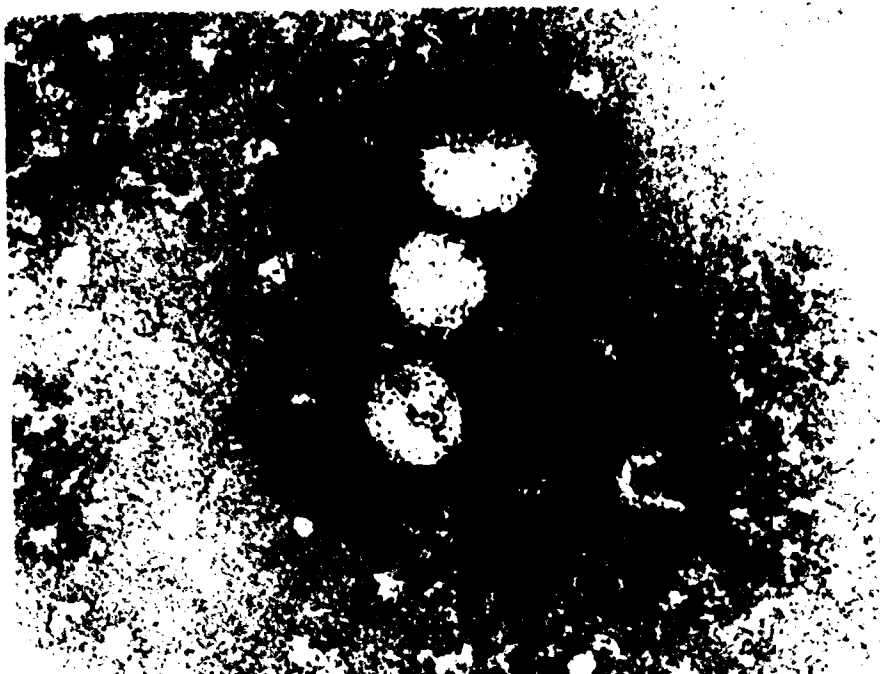
FIGURE 1
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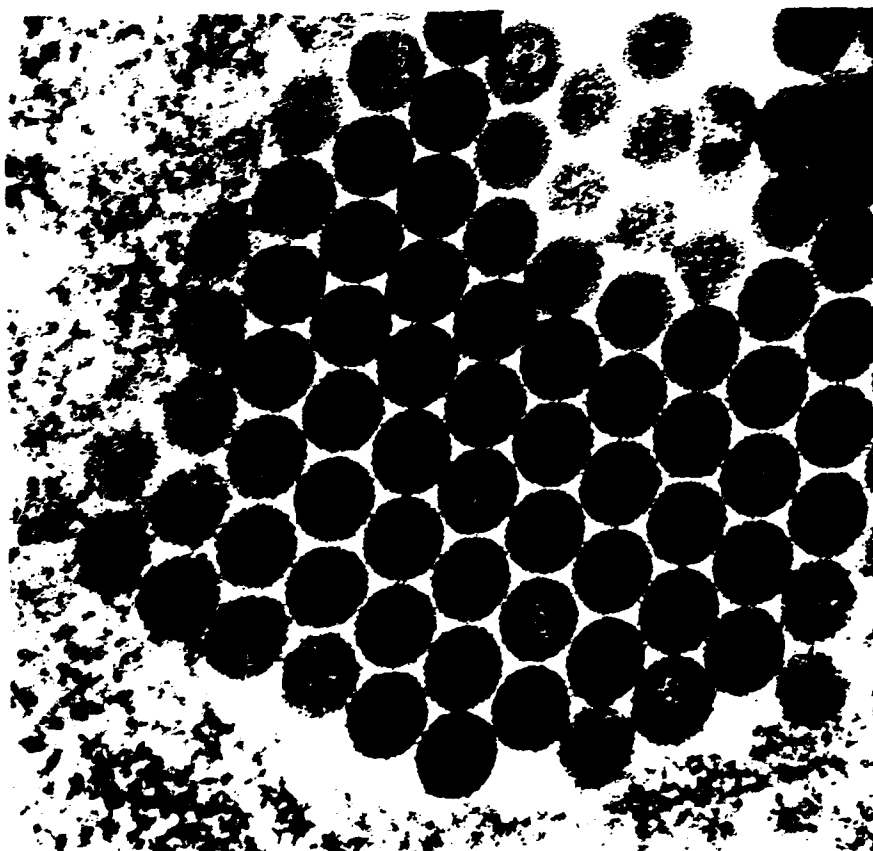
KARIMABAD VIRUS

FIGURE 2
(X 105,000)
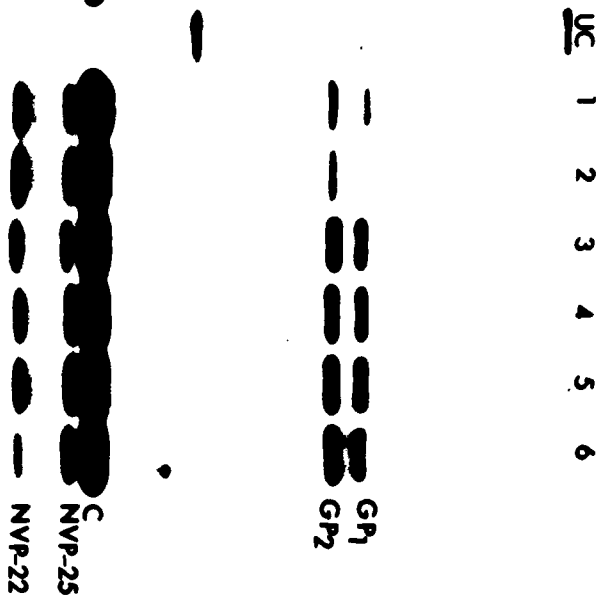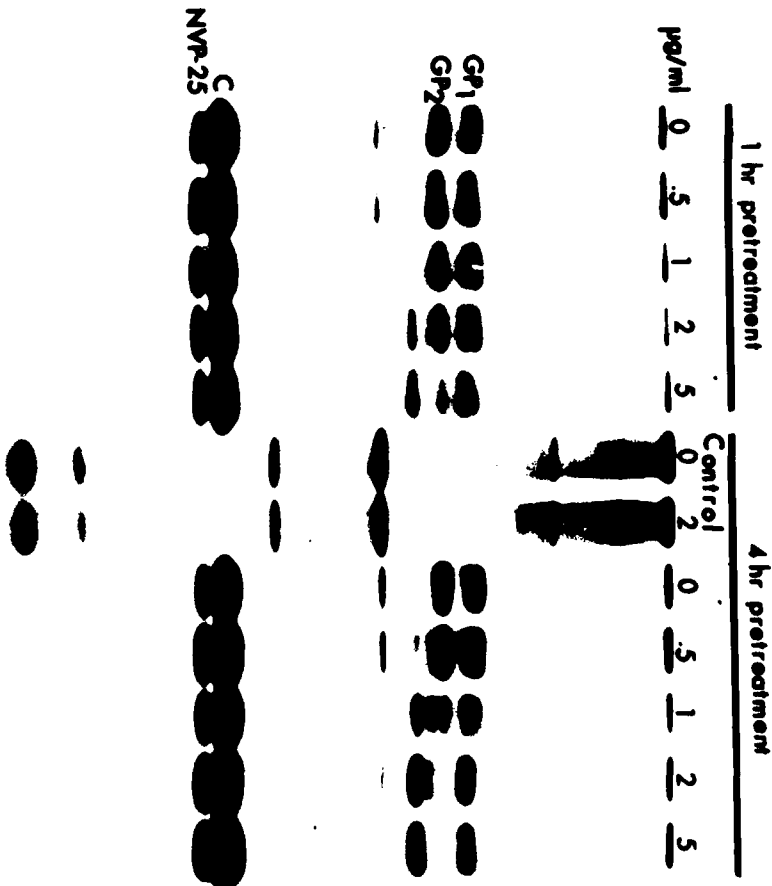
FIGURE 3

Immunoprecipitation Analysis of 6 PTV Strains

Figure 4
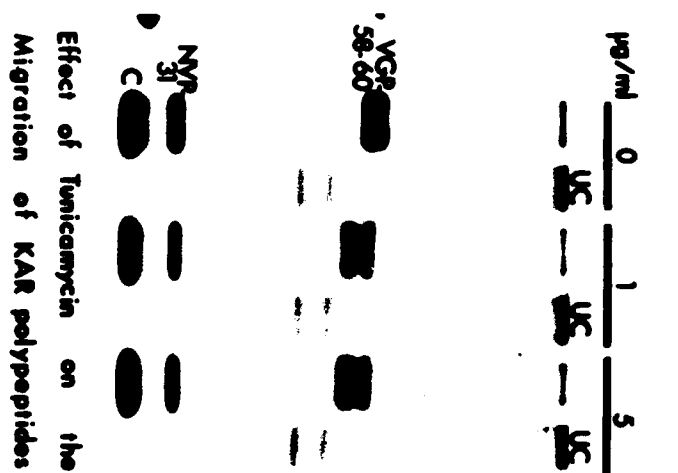
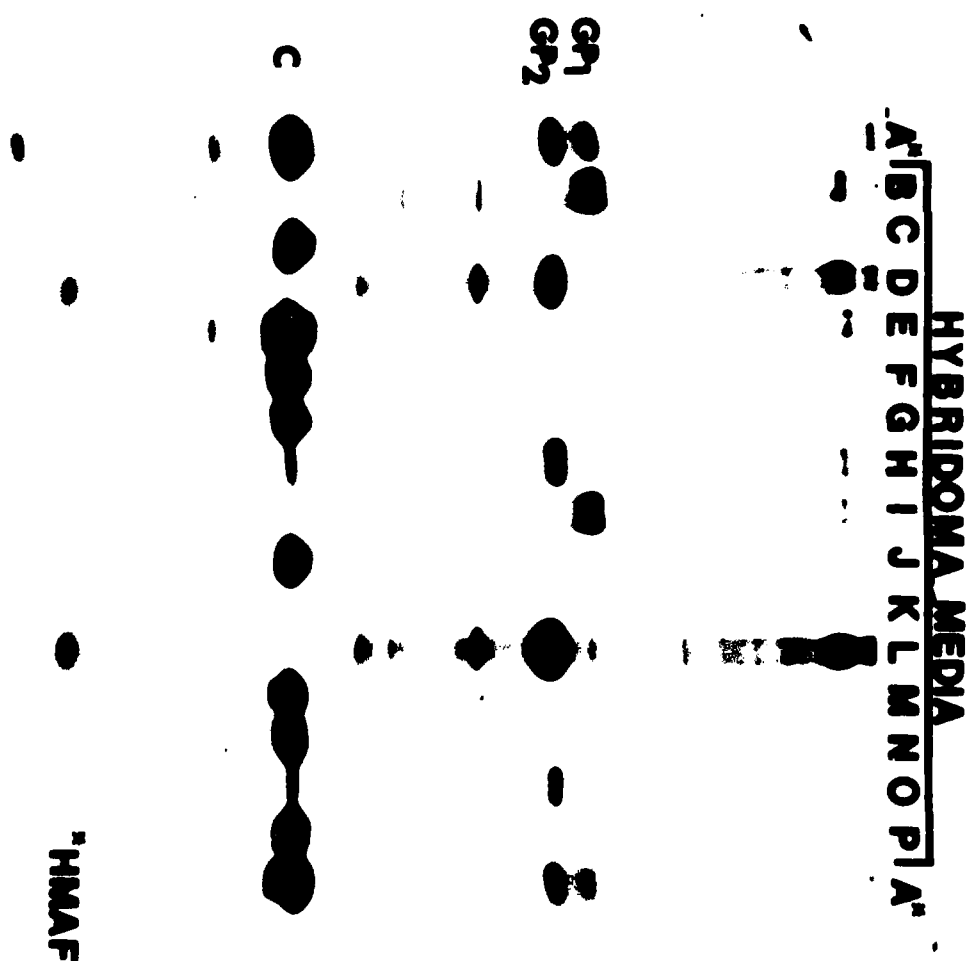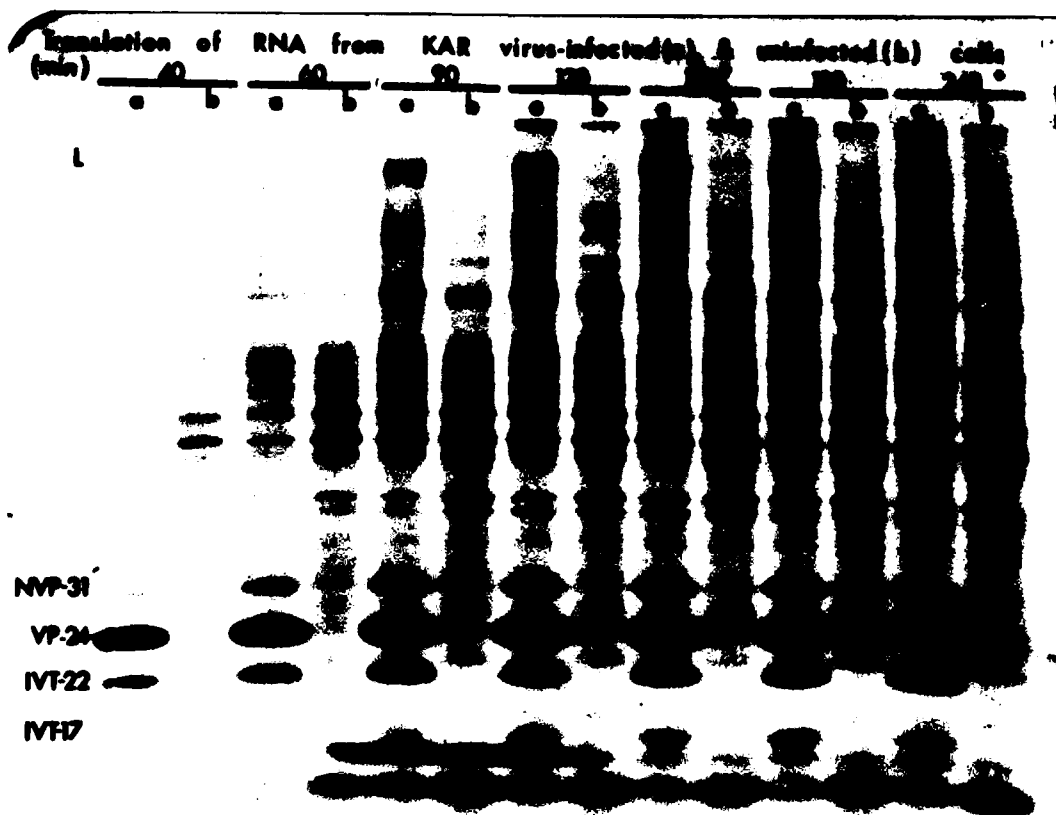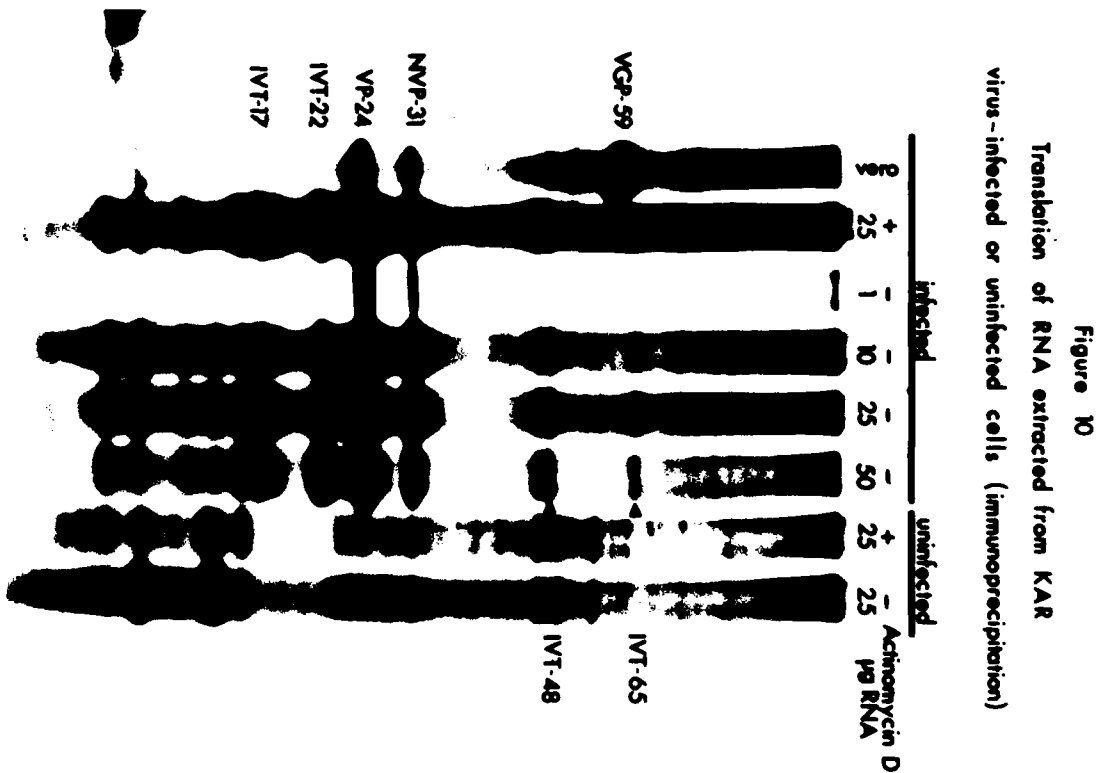Effect of Tunicamycin on the Migration of PTV Polypeptides

Effect of Tunicamycin on the
Migration of KAR polypeptides

Figure 5

figure 6

HYBRIDOMA MEDIA

"HMAF"

NVP-31
VP-24
IVT-22
IVT-17

FIGURE 9

Figure 9

Immunoprecipitation of KAR
Polypeptides synthesized in vitro

Figure 10
Translation of RNA extracted from KAR
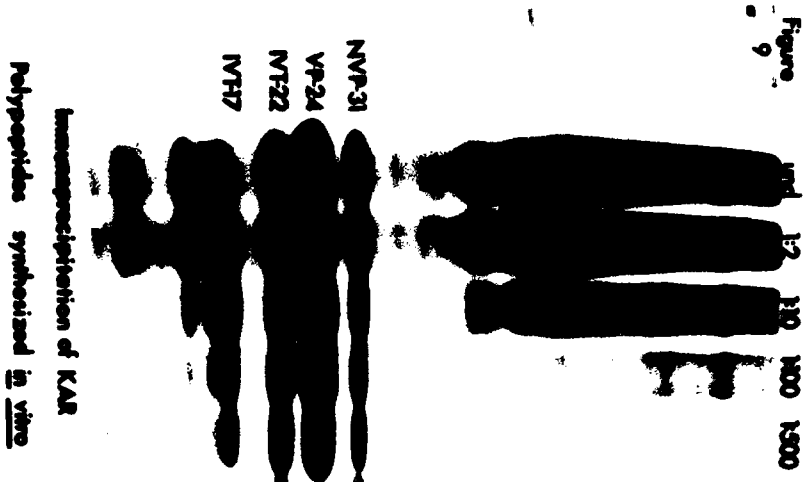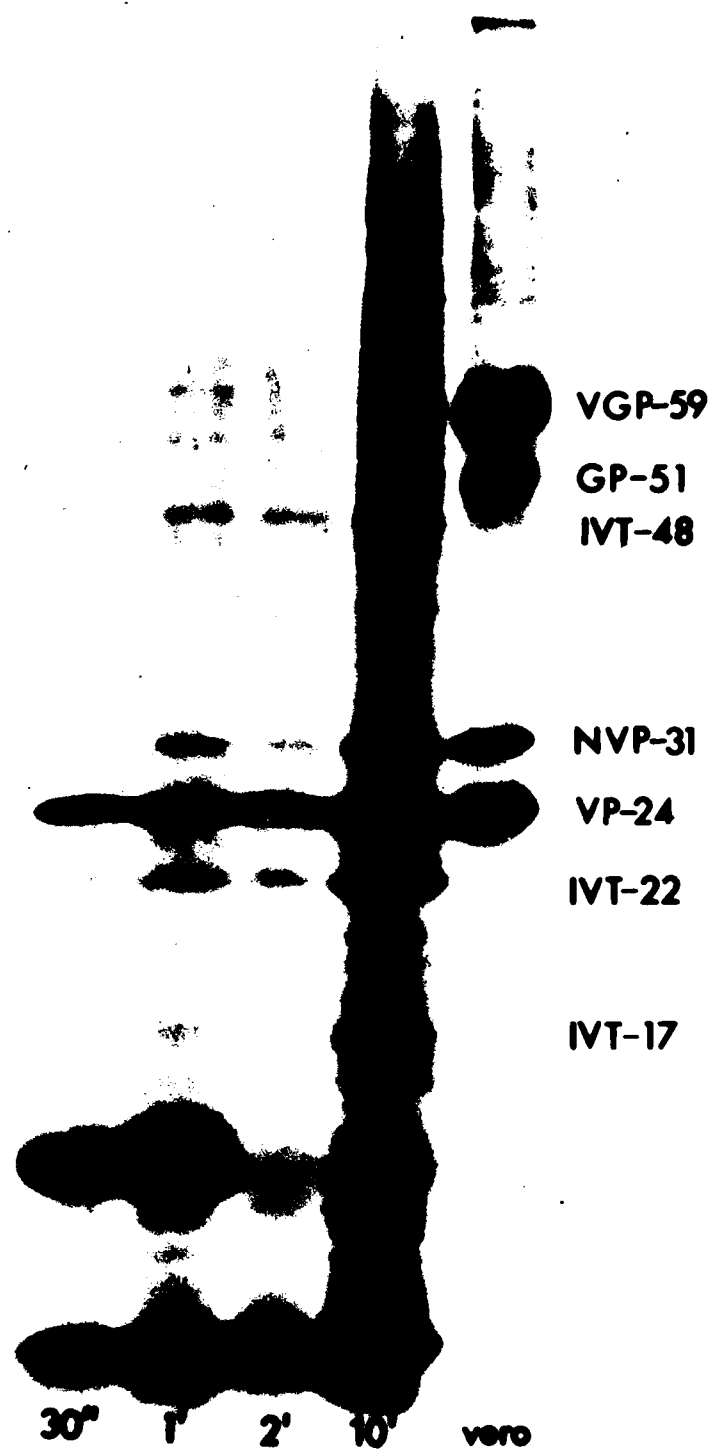virus-infected or uninfected cells (immunoprecipitation)

Pulse Labelling of KAR polypeptides

Figure 11

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